UNIVERSITY OF SOUTHAMPTON

THE ROLE OF GASTRIN AND CCK-B/GASTRIN RECEPTOR IN HEPATOCELLULAR AND PANCREATIC CANCERS

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DEDICATION

This thesis is dedicated to my wife Jackie
and children - Arthur and Freddie

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE

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THE ROLE OF GASTRIN AND CCK-B/GASTRIN RECEPTOR IN HEPATOCELLULAR AND PANCREATIC CANCERS

Gastrin acts as a growth factor in the normal gastrointestinal tract and is able to promote the growth of both gastrointestinal and non-gastrointestinal cancers. Following post-translational processing, the precursor forms of gastrin, including progastrin and glycine-extended gastrin, as well as the fully processed amidated gastrin, are expressed by tumours at variable concentration. Each gastrin form is a potential mitogen.

The *in vitro* effects of gastrin, glycine-extended gastrin, anti-gastrin antibodies and the CCK-B receptor antagonist PD135,158 were assessed on hepatoma cell lines. This study demonstrated that amidated gastrin and glycine-extended gastrin stimulated some hepatoma cell lines and that this could be abrogated by anti-gastrin agents.

Tissue sections from patients with hepatocellular carcinoma, fibrolamellar carcinoma, cholangiocarcinoma as well as normal liver biopsies were assessed for expression of CCK-B receptor and gastrin isoforms. The results showed that most liver tumours express CCK-B receptor and precursor forms of gastrin. There appears to be little expression of the receptor and no expression of precursor forms of gastrin in normal liver.

Tissue sections from patients with pancreatic cancer and normal pancreas were assessed for expression of CCK-B receptor and gastrin isoforms. The results showed that the normal pancreas showed no expression of receptor or gastrin isoforms except for occasional cells in the islets. The pancreatic cancer patients showed definite expression of CCK-B receptor and predominantly precursor forms of cancer.

Studies were performed to identify the cellular sites of expression of the CCK-B receptor in the known CCK-B receptor bearing pancreatic acinar AR42J cells. Using immunoelectron microscopy and western blotting techniques the CCK-B receptor was shown to be expressed not only on the cell membrane, but also in the cytoplasm and nucleus of cells.

In conclusion the expression of the gastrin precursor forms is most likely related to the autocrine production of gastrin by cancer cells. The nuclear expression of the receptor is a novel finding and may contribute to cellular proliferation in cancer cells. Gastrin and its receptor appear to be important in the growth of a variety of cancers and the understanding of this proliferative effect has lead to the development of anti-gastrin agents now undergoing therapeutic clinical trials in gastrointestinal and pancreatic cancers.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1:

INTRODUCTION

Contents

1.1 Background

In 1905 Edkins demonstrated that the injection of pyloric membrane extracts into anaesthetised cats resulted in gastric acid secretion. Edkins postulated that the pyloric membrane extract contained a stimulatory substance, which he called gastric secretin or gastrin (1, 2). This was a challenge to the Pavlovian doctrine of "nervism" as Pavlov had shown in 1895 that gastric secretion was controlled by neural mechanisms and it was therefore thought that nerves performed all gastrointestinal secretory functions (3). This puts in to context the enormity of the discovery of the first "hormone" by Bayliss and Starling in 1902 when they demonstrated that pancreatic secretion could be stimulated by a chemical secretagogue, secretin (4, 5).

In 1910 Dale and Barger discovered histamine (6) and it was subsequently demonstrated that histamine was a potent stimulant of gastric acid secretion (7). As a result of the evidence for the role of histamine in gastric acid secretion, many now questioned the nature of Edkins hormone gastrin and there were vociferous claims especially from Ivy's laboratory in Chicago that Edkins hormone was in fact a histamine contaminant within the pyloric extract (8). It was not until 1938 when Komarov isolated gastrin that the unique identity of Edkins hormone was accepted and by the time this was published in 1942 Edkins had died (9).

The next major advance in the history of gastrin was in 1964 when Gregory and Tracey isolated gastrins I and II, unsulphated and sulphated gastrin-17 respectively. The following year they determined the active site of gastrin as the carboxy (C) – terminal residues Trp-Met-Asp-Phe-NH2 (10). 1n 1970 Yalow and Berson discovered that there was a "bigger" form of gastrin when they identified gastrin-34 (11). Two years later Rehfeld using chromatographic techniques,

demonstrated that there were many forms of circulating gastrin both longer and shorter than the known gastrins 17 and 34 (12).

1.2 Post-translational Processing of Gastrin

In recent years the post-translational processing of gastrin has become better understood. This process is crucial to the determination of size of gastrin and biological function.

The post-translational processing mechanisms involved in the production of "mature" amidated gastrin in the antral G cells is predominantly an enzyme driven cascade (see fig.1). Recently some of the intermediate forms have been shown to possess biological activity. The human gastrin gene consists of three exons and encodes a single 0.7kb mRNA producing preprogastrin, a precursor peptide of 101 amino acids (13). Preprogastrin is translocated to the endoplasmic reticulum where the N-terminal signal sequence is cleaved at residue 21 by a signal peptidase to produce progastrin. This passes to the Golgi apparatus for O-sulphation and endoproteolytic prohormone convertase cleavage. Vesicles carry the resulting intermediate peptides toward the basal aspect of the G cells, where they are stored in secretory granules (14, 15). Processing probably continues during this transport, with further cleavage and glutaminyl cyclization of the N-terminus to producing glycine-extended gastrin-17 and glycine-extended gastrin-34 (G17-gly & G34-gly) (16, 17, 18). The final processing steps occur in the granules, where the amidation enzyme complex peptidylglycine α-amidating monoxygenase removes glyoxylate from glycine-extended gastrin forming bioactive α-carboxyamidated peptides, which are stored for subsequent release (19, 20). As a result of this complex processing, the G-cells release a mixture of gastrin peptides from the secretory granules. Most of the

peptides secreted are amidated gastrins. The predominant form of amidated gastrin (G-NH2) is gastrin-17 (G17), with some gastrin-34 (G-34) and small amounts of other gastrin peptides (gastrin -71, -52, -14 and shorter fragments) being released (21, 22). Non-endocrine cells (which lack the full processing machinery) will secrete a higher proportion of precursor gastrin forms, as will endocrine cells with an increased gastrin synthetic rate (as certain enzymes cannot keep pace with the maturation process) (23). For example, compared with the gastric antrum, colorectal cancer cells produce a higher proportion of progastrin species and less fully processed gastrins, and these forms are more likely to be secreted and less likely to be stored (24, 25, 26, 27). The effect of the precursor forms is enhanced by their slower clearance from the circulation.

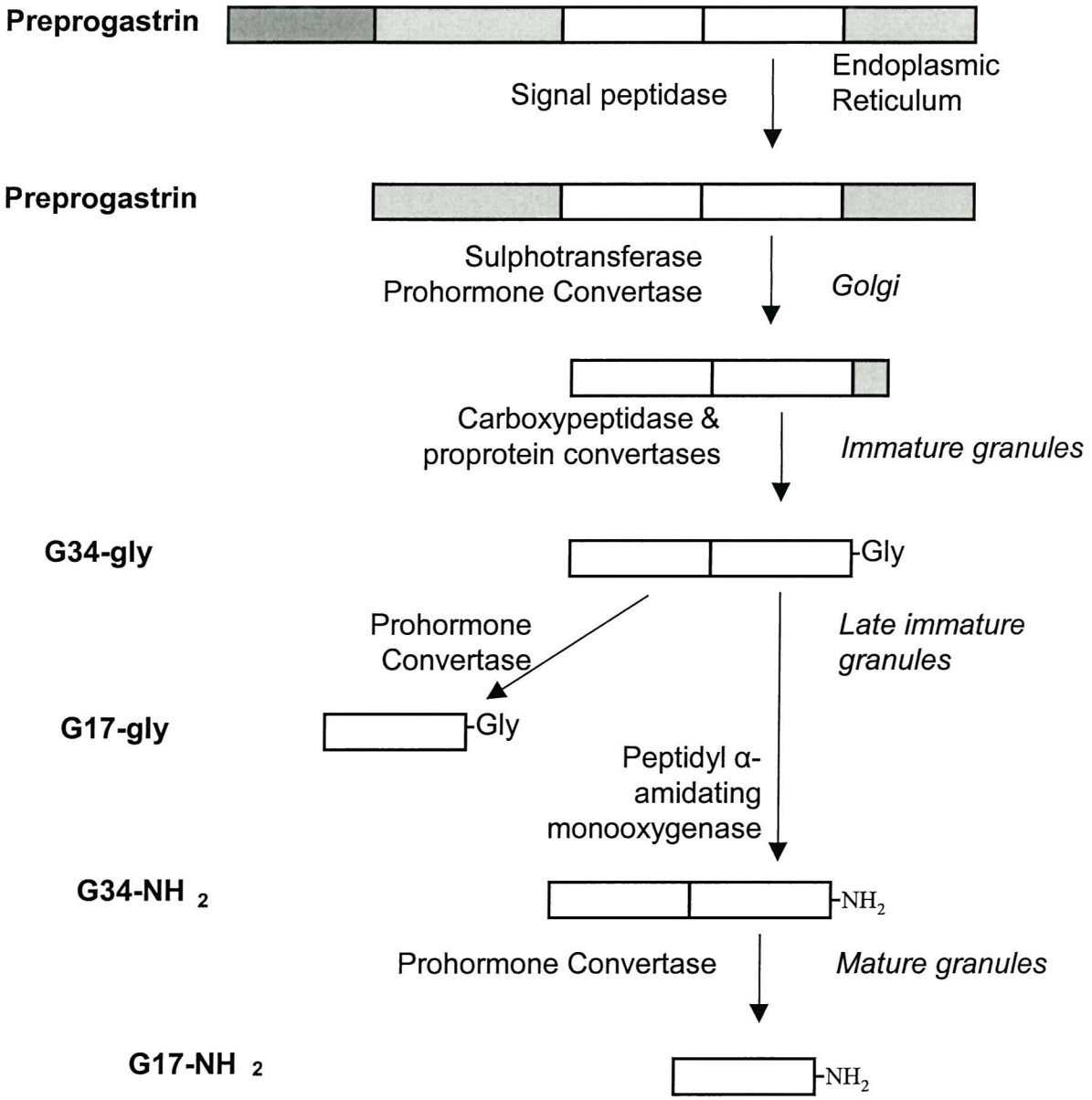


Figure 1. The post-translational processing of Gastrin. Gly-G17, G34-NH2 and G17-NH2 are secreted from the G cell, and with the exception of preprogastrin, all other forms may be secreted from cancer cells. See text for details.

G34-gly = glycine-extended gastrin 34, G34-NH2 = amidated gastrin 34,

G17-gly = glycine-extended gastrin 17; G17-NH2 = amidated gastrin 17.

1.3 CCK-B/Gastrin Receptor

The "mature" amidated gastrin binds to the Cholecystokinin type B (CCK-B) receptor (28, 29) whereas the precursor forms of gastrin bind to an as yet uncharacterised receptor, which may turn out to be a splice variant of the CCK-B receptor (30).

The CCK-B receptor belongs to a family of G protein coupled receptors with seven transmembrane domains that have equal affinity for both CCK and gastrin (31). This receptor was named CCK type-B receptor as it was found predominantly in the brain (32). It was subsequently found to be identical to the peripheral CCK-B receptor in the parietal and enterochromaffin-like cells of the stomach (33). This receptor has been well characterised in a number of normal (34, 35) and tumour tissues (36, 37) and studied extensively using the rat pancreatic adenocarcinoma cell line AR42J (38). The AR42J CCK-B receptor cDNA is more than 90% homologous to that in rat and human brain and more than 84% homologous to canine parietal cell CCK-B receptor cDNA (39), demonstrating a high sequence homology even between species. In man there is 48% homology with the CCK type-A (abdominal) receptor, which is predominantly localised to the gallbladder, pancreas, spinal cord and hypothalamic nuclei (32).

In terms of ligand binding to the CCK-B receptor it has classically been considered that peptide hormones bind to surface membrane receptors with the resulting formation of secondary messengers regulating cell function (40). The binding of gastrin to the CCK-B receptor leads to breakdown of phosphatydyl inositol, and protein kinase C activation with a resultant increase in intracellular calcium ion concentration, as well as the induction of c-fos and c-jun genes via the mitogen-activated protein kinase - which has been implicated in the regulation of cell proliferation (41). Additionally, gastrin binding to the CCK-B receptor has been associated with the subsequent increased

phosphorylation of a tyrosine kinase, pp125FAK (focal adhesion kinase), which may also have a role in the transmission of mitogenic signals (42).

1.4 CCK-B/Gastrin Receptor Isoforms

Two different mRNA products from the CCK-B receptor gene have been identified and result from different splicing within a region of exon 4 (43). The products of these mRNAs differ in the third intracellular loop of the protein by 5 amino acids. It is presumed that the differences in the receptors result in different signal transduction upon binding of CCK or gastrin to the receptor. The shorter form predominates in parietal cells, and although the majority of the CCK-B receptor in colorectal cancer cells is also the short isoform (44/54 cases), the long isoform is also present in 17/54 cases (44).

Studies of the variants in transfected cell lines showed no significant difference when assessed by agonist affinity, signal transduction by intracellular calcium concentration, activation of the mitogen-activated protein kinase or focal adhesion kinase pathways and the induction of the early response genes *c-fos* and *c-myc* (45). The intracellular calcium increase is more sustained upon ligand binding to the long form of the receptor (44), so it seems that the short form (present on the parietal cells) is less likely to demonstrate persistent activation with low levels of circulating gastrin.

ΔCCK-B is a further splice variant generated by use of a novel exon and lacks the amino terminal extracellular domain. This truncated receptor differs in ligand selectivity, binding G17 with a lower affinity and CCK with a higher affinity. It is co-expressed with CCK-B in the normal fundus and brain, but exclusively expressed in AGS gastric tumour cells, suggesting a potential for tissue specific expression (46). It

has also been shown in 3/5 gastric and 5/8 colorectal cell lines where it was co-expressed with gastrin (47).

1.5 Other Gastrin receptors

Another receptor has been identified, but its role is less clear. It may be the progastrin receptor. It is a 78-kDa protein, originally termed the gastrin-binding protein (GBP), and is related to the family of enzymes possessing enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities. It has been isolated from various tissues, including human gastric cancer cell lines and porcine gastric mucosa (48, 49). GBP recognises both the N- and C-termini of gastrin17, with a low affinity for amidated gastrin and G17-gly. Progastrin affinity is hard to measure because of the difficulty in synthesis of the protein, but as C-terminal amidation is not a prerequisite for binding of gastrin to the GBP, it is a potential target for progastrin. There is a high concentration of progastrin within tumour cells, and this would mean that GBP could function as part of an autocrine pathway even with a low affinity for progastrin (50). GBP mRNA was found in 100% of colorectal cancer using RT-PCR (44), and this group has confirmed the binding of progastrin to the receptor. Many feel that this evidence is enough to propose GBP as the putative "CCKC" receptor, but the view is not universally held. Other groups point to the ubiquity of the protein and the low affinity for gastrin as evidence against its role (51).

Another putative receptor, with a high affinity and selectivity for gly-G17, has been described on rat pancreatic AR42J cells. The binding of glycine-extended gastrin to these cells and its proliferative effects were not stopped by blockade of the CCK_2, and therefore are not mediated through this receptor. This G17-gly receptor requires both the N- and C-terminal portions of G17-gly for optimal binding and both high and

low affinity receptor subtypes exist which by cross-linking studies appear to be proteins of differing sizes (52).

Recently a novel splice variant of the human CCK-B receptor was identified which was generated by intron 4 retention during RNA processing. The resulting receptor protein contains 69 additional amino acids residues in its third intracellular loop domain. Expression of this novel splice variant (designated CCK-BRi4sv for intron 4 containing splice variant) was detected in human colorectal cancers and adenomatous polyps but not in normal colon adjacent to the cancer (153). The properties of this receptor suggests that it may stimulate cancer cell proliferation through a gastrin-independent mechanism.

1.6 Gastrin as a Growth Factor

The earliest demonstration of the trophic effect of gastrin was in 1969 when Crean et al demonstrated that the parietal cell mass increased after the chronic administration of pentagastrin to rats (53). The following year it was demonstrated that removal of the rat antrum (containing the G cells) resulted in atrophy of the fundic mucosa but that this atrophy could be reversed by administration of pentagastrin. The trophic action was not a result of gastrin as Williams et al showed that gastrin stimulated DNA synthesis in canine oxyntic glands but that histamine had no effect (54). Other studies examining the ıcorporation of ^{3}H-thymidine have demonstrated that gastrin infusions increase the p ›liferation of cells in the neck of the oxyntic glands, where stem cells reside (55). Th se effects of gastrin have also been shown by work in transgenic mice over-exp ssing amidated gastrin (INS-GAS), where there was an 85% increase in cells unde oing proliferation in the neck of the oxyntic glands (56). In contrast, Gastrin-knockc ıt mice have a decrease in parietal and ECL cell mass, but the basal fundal prolifera ion rate remains normal (57, 58). This suggests that while gastrin may not be essential ı ›r maintaining the basal rate of proliferation in the fundus, it does affect the maturation ›f cells in the neck of the gastric glands and influences their differentiation to either parietal or ECL cells. These mice also showed reduced proliferation of the colonic mucosa. Recei tly glycine-extended gastrin was shown in gastrin deficient mice to produce a 10% increase in the thickness of colonic mucosa and an 81% increase in colonic proliferation when compared to saline infusion (59). High serum progastrin (with normal amidated gastrin) in transgenic

mice results in a marked increase in the bromodeoxyuridine labelling index of the colon compared to gastric mucosa, this effect was not seen in control mice (56).

One of the earliest demonstrations of the trophic effects of gastrin in man was the observation in patients with Zollinger-Ellison syndrome that they had increased proliferation of the oxyntic mucosa (60). In 1993 Sobhani found that patients with Zollinger-Ellison syndrome had colonic mucosal hypertrophy (61).

1.7 Gastrin and Tumour Growth

The earliest association of gastrin and tumour growth was in the early 1980's at the time of the development of proton pump inhibitors (PPIs). It was noticed that long term administration of PPIs resulted in enterochromaffin-like (ECL) cell proliferation and after life-long inhibition of gastric acid secretion carcinoid tumours developed in the stomach of rats (62). Specific CCK-B/gastrin receptor antagonists were able to inhibit the proliferative process. Further evidence for the involvement of gastrin and CCK-B receptor in ECL cells comes from the African rodent *Mastomys natalensis* which is genetically susceptible to ECL gastric carcinoid tumours. The rodents develop tumours which are stimulated by physiological concentrations of gastrin, perhaps because of spontaneous activation of the CCK-B/gastrin receptor (63). CCK-B receptor antagonists have been shown to inhibit the growth of transplanted ECL tumours from *Mastomys* (64).

An association of tumour growth with hypergastrinaemia in man, was first described in 1993, by Sjoblom et al, who demonstrated that patients with long standing pernicious anaemia developed gastric carcinoid tumours (65). However in man the situation regarding hypergastrinaemia is probably more complex. Only 1% of patients with Zollinger-Ellison syndrome develops carcinoid tumours in the absence of multiple

neoplasia type-1 (MEN-1) despite markedly elevated serum gastrin. In the presence of MEN-1 13% to 30% of patients develop tumours (66). This would suggest that the tumour development is related to the genetic instability of MEN-1 and hypergastrinaemia. Currently there is just one report of high dose anti-secretory therapy being associated with the development of gastric carcinoid in man (67). In man other factors may also be required for the development of carcinoid tumours such as smoking or *Helicobacter pylori* infection.

The first conclusive demonstration of the trophic effects of gastrin in cancer proliferation was in colorectal cancer. In 1986 CCK-B/gastrin receptor was isolated from human colorectal cancer (68) and subsequent *in vitro* (69, 70, 71, 72, 73) and *in vivo* (74, 75, 76, 77) studies demonstrated that not only amidated gastrin but also precursor glycine-extended gastrin were potent mitogens. Additionally anti-gastrin agents were shown to abrogate these proliferative effects (78).

An autocrine pathway for the expression, processing and secretion of gastrin was first suggested in human colon cancer by Kochman in 1992 (79). Other recent series have shown divergent results for amidated gastrin (8-100%) and glycine-extended gastrin (0-100%) with a consensus of the majority of patients expressing progastrin (87%-100%) (80, 81, 82). The secretion of amidated gastrin forms implies the expression of all the processing enzymes within the cell, as discussed above, and increased quantities of precursors will be secreted from cells which do not have the full complement of enzymes to process gastrin to its "mature" amidated form.

With the known expression of gastrin forms by colon cancer cells it has been suggested that one of the events in the adenoma carcinoma sequence is the activation of the gastrin gene. The evidence from animal and human studies is that this gastrin gene activation is an early step. In two animal models of polyposis coli with a mutant

adenomatous polyposis coli (APC) gene, gastrin expression was seen in colonic polyps and not in the control animals (83). In man it was also shown that 80% of colonic adenomas express gastrin, including polyps of less than 1cm (84). In the Fearon & Vogelstein multigenetic model for sporadic colorectal tumourigenesis it has been proposed that the gastrin autocrine pathway is activated early at the hyperproliferative epithelium stage on the path to tumourigenesis (85).

In recent years there has been a strong body of evidence for the trophic role of gastrin in gastric cancer. As for colon cancer, both *in vitro* and *in vivo* studies have demonstrated the proliferative effects of amidated and precursor forms of gastrin with anti-gastrin agents able to abrogate these proliferative effects (+).The key study in defining the effect of gastrin promoting gastric cancer was the transgenic INS-GAS mouse model where mice overexpressed amidated gastrin (154). Circulating gastrin levels were approximately twice that of "control" wild type mice. In addition to thickening of fundic mucosa the overexpressing transgenic mice initially developed proliferation of parietal cells and ECL cells however over time they subsequently developed parietal cell loss, gastric atrophy, foveolar hyperplasia and gastric metaplasia. After more than twenty months the majority of INS-GAS mice developed gastric carcinoma (154). A recent review of human Gastric cancer resection samples demonstrated that the gastric cancers expressed gastrin mRNA suggesting a gastrin autocrine pathway (86). Gastrin expression has also been noted in some pre-malignant stomach pathology (87).

There is less evidence for other cancers but gastrin and its precursor forms have been shown to promote the growth of pancreatic carcinoma (88), hepatocellular carcinoma (89), renal cell carcinoma (90) and small cell carcinoma of the lung (91).

1.8 Thesis in context of current understanding

The aim of this thesis is to provide more evidence for the role of gastrin in those cancers with currently limited evidence, such as primary liver cancers and pancreatic cancer. Additionally in view of an unexpected finding of nuclear expression of an anti-CCK-B/gastrin receptor antibody in cancer cells, I also examined the translocation of this receptor antibody in a cancer cell line.

Chapter 2:

EFFECT OF GASTRIN AND ANTI-GASTRIN ANTIBODIES ON THE PROLIFERATION OF HEPATOCYTE CELL LINES

Contents

2.1 Introduction

There has been little study on the effect of gastrin in the liver, which is surprising when one considers that gastrin passes from the antrum and duodenum to the liver via the portal venous system. It has been shown that smaller forms of gastrin, probably less than seventeen peptides are inactivated on transit through the liver (92, 93). More recently the CCK-B/gastrin receptor has been identified on normal mouse hepatocytes and gastrin shown to promote the growth of these hepatocytes in the presence of epidermal growth factor (94). Further animal studies have shown that gastrin may be involved in liver regeneration (95) although other studies have not supported this (96). Recently it has been shown that gastrin acts as an inhibitory hormone in the proliferation of cholangiocarcinoma (97). The biological significance of gastrin in liver and liver tumours therefore remains to be determined.

The purpose of this present series of experiments was to investigate the effect of gastrin-17 and glycine-extended gastrin-17 on liver tumour and embryonic liver cell lines. This study is part of a longer-term strategy to determine the clinical relevance of gastrin in both normal and cancer cell populations. In order to assess the possible therapeutic role of anti-gastrin agents in "gastrin sensitive tumours", the effect of a specific CCK-B/gastrin receptor antagonist as well as anti-gastrin antibody was investigated in this *in vitro* study.

2.2 Materials and methods

2.2.1 Cells

The cell lines AR42J, MCA-RH 7777, PLC/PRF/5, HepG2 and WRL68 were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK). The cell culture media Dulbecco's Modified Eagles Medium (DMEM), Eagles

Minimum Essential Medium with Earles Balanced Salt Solution (EMEM) and RPMI-1640.

Medium was obtained from Sigma Chemical (Poole, UK). Foetal calf serum (FCS) and non-essential amino acids (MEM) were obtained from Life Technologies (Paisley, UK). Synthetic human gastrin I (G17), L-glutamine, EDTA 0.02%, trypan blue, propidium iodide and thymidine were obtained from Sigma Chemical (Poole, UK). Synthetic glycine-extended gastrin 17 (G17-gly) was a gift from Aphton Corporation (Woodland, CA). The specific CCK-B/gastrin receptor antagonist PD135,158 was obtained from RBI, Massachusetts.

2.2.2 **Cell Culture**

AR42J derived from a rat pancreatic adenocarcinoma (98) and known to be a gastrin-sensitive cell line, was used as a positive control (passage nos. 12-40) (99). It was cultured in RPMI-1640 medium containing 10% FCS and 2mM glutamine. PLC/PRF/5 hepatoma cell line (passage nos. 60-80) derived from a patient with primary liver cancer who was Hepatitis B surface antigen positive, and HepG2 cell line (passage nos. 94-110) derived from a child with primary hepatoblastoma were grown in DMEM containing 10% foetal calf serum and 2mM L-glutamine. MCA-RH7777, a rat hepatocellular carcinoma cell line (passage nos. 78-92) was cultured in DMEM containing 10% FCS with 2mM L-glutamine and 1% non-essential amino acids. WRL68, a human embryo liver cell line (passage nos. 92-106) was cultured in EMEM containing 10% FCS and 2mM L-glutamine.

All cells were maintained at 37°C in 5% CO_2 in air at 100% humidity and were grown to 80% confluence in T75 flasks (Falcon, London, UK) and passaged following 0.02% EDTA to bring adherent cells into suspension.

Twenty-four hours prior to each experiment, cell lines were changed to serum free culture media (S-Fm). Only cell population with a viability of above 90% were used. The viability of the AR42J cell line grown in S-Fm was less then 80% as determined by trypan blue dye exclusion. For AR42J cells viability greater than 95% could be achieved with 1% FCS which was therefore used. The growth of cells was synchronised by the addition of 1mM Thymidine to the medium. FACS analysis (see below) was performed to ensure that all cells were in G_1 phase at the start of the experiment.

1 x 10^4 cells of each cell line in 90ul serum free (1% FCS for AR42J) media were dispensed into each well of a 96 well plate (Abbott, Maidenhead, UK). Cells were allowed to settle for four hours then 10ul of either G17 or G17-gly in S-Fm at final concentrations ranging from 10^{-6}M to 10^{-11}M was added. In the control well, S-Fm (1% FCS for AR42J) without G17 or G17-gly was added. Parallel experiments were performed to assess the inhibitory effect of (i) the CCK-B/gastrin receptor antagonist PD135,158 at a concentration of 10^{-4}M (100) and (ii) excess anti-G17 (0.9 mg/ml) specific for the N-terminal end of G17 and therefore immunoneutralising both G17 and G17-gly. Control experiments were performed using preimmunised purified rabbit IgG.

2.2.2 **Antibodies**

Anti-gastrin (anti-G17) Antibodies

Anti-gastrin antibodies were raised, by Aphton, USA, in rabbits by an immunogen G17DT (Gastrimmune^{TM}), previously described (72, 78). Briefly, G17DT is a water-in-oil emulsion containing a synthetic target peptide epitope from the N-terminal sequence of human gastrin-17 molecule conjugated with a 7 residue spacer to modified purified diphtheria toxoid with 6-maleimido caproic acyl N-hydroxy succinimide (MCS) ester (Sigma Chemical, US) and normuramyl dipeptide adjuvant. For experiments involving the rat cell lines AR42J and MCA-RH7777 a rat G17DT was similarly synthesised also targeting the N-terminal sequence of rat

gastrin-17. The appropriate immunogen was administered to New Zealand white rabbits every four weeks and sera obtained after three immunisations. The antibodies were subsequently prepared by affinity purification over a column comprising the amino terminal 9 residue sequence of G17 bearing a C-terminal Lys coupled to aminolink resin (Pierce Chemical Co. USA).

CCK-B/gastrin Receptor Antibody

The CCK-B/gastrin receptor antibody, a gift from Aphton, USA, is an affinity purified rabbit polyclonal antibody raised against the amino-terminal sequence of the human CCK-B/gastrin receptor, as previously described (78). Briefly, peptide with the sequence KLNRSVQGTGPGPGASLSSPPPPC, comprising residues 5-21 of the human CCK-B receptor followed by a seven residue spacer at the C-terminus was custom synthesised (BACHEM, Torrence, CA, USA). The peptide linked to modified purified diphtheria toxoid (DT) with MCS. Immunogens were formulated at 1.0mg/ml conjugate by emulsifying conjugate-bearing aqueous phase with Montanide ISA 703 adjuvant (Seppic, Paris, France) in a 30:70 (wt: wt) ratio of water : oil. New Zealand white rabbits were injected intramuscularly with 0.25ml/injection at 0,4 and 8 weeks Serum was prepared from blood collected subsequent to the third injection, and the immunoglobulin fraction was prepared by affinity purification of the sera over recombinant Protein A-Agarose (Boehringer Mannheim GmbH, Germany).

2.2.4 Immunocytochemistry

Cytospins of each cell line, 10^5 cells/slide, were air-dried and fixed in cold acetone (4°C) for 5 mins. The CCK-B/gastrin receptor was identified using the rabbit polyclonal antibody at a dilution of 1:100 incubated for 1hr at room temperature. A standard indirect goat-ant-rabbit alkaline phosphate method was used to visualise

antibody binding. Immunohistochemical controls included absorption of the antibody with purified CCK-B/gastrin receptor, and omission of the antibody in the immunostaining procedure.

2.2.5 **FACS Analysis**

FACS analysis was performed to verify cell synchronisation by determination of DNA content. Each cell line was assessed independently. Twenty-four hours prior to analysis each cell line was incubated in serum free media (except AR42J, 1% serum in media). The cells were treated with either 1mM thymidine in media or media alone. Cells were centrifuged at 400G for 6 mins, resuspended in 1.5 ml 90% ethanol and stored at –20°C until analysis. Cell were then washed twice in Phosphate Buffered Saline pH 7.4 (PBS) and resuspended in 300µl of PBS containing 5µg/ml propidium iodide (Sigma) and 50ng/ml heat treated RNAse in PBS. Flow cytometry was performed on a FACScan flow cytometer (Beckton Dickinson, San Jose, CA). Viable cells were gated and doublets excluded. Scans were analysed using Lysis II software.

2.2.6 **Proliferation Assay**

The CellTiter 96 non-radioactive Aqueous Assay was used (Promega, Southampton, UK) to measure cellular proliferation at 48 hours, this being the peak time of cell viability and proliferation (data not presented). The assay is based on the production of a soluble coloured formazan from a tetrazolium compound, monotetrazolium salt (MTS) by viable cells. The absorbance at 490nm was determined using a plate reader (Abbot, UK) and was directly proportional to the number of living cells. Cell proliferation was determined by expressing absorbance

values from G17 or G17-gly stimulated cells as a percentage of unstimulated control values.

2.7 Statistical Methods

All Values are expressed as mean of percentage absorbance +/- S.E.M. Statistical evaluation was performed using Student's *t* test.

2.8 Results

2.8.1 FACS Analysis

All cell lines were synchronised in G_1 phase prior to each experiment (figures 2a and 2b).

2.8.2 G17 Stimulation

AR42J cells were used as a positive control. G17 at concentrations of 10^{-9} to 10^{-11} M caused a significant growth of proliferation of these cells. There was a maximum rise of 48.3% at 10^{-10} M for G17 ($p < 0.001$) at 48h compared to unstimulated control cells (figure 3a). At a concentration of 10^{-6} M G17 there was significant inhibition of growth of the AR42J cells ($p < 0.001$). Anti-G17 also inhibited the proliferative effect of exogenous G17 at all concentrations. The addition of PD135,158 caused 23.4% inhibition of unstimulated AR42J cells ($p < 0.001$). PD 135, 158 inhibited the proliferative effect of G17 except at 10^{-6} M where the excess G17 overcame the inhibitory effect.

G17 at concentrations or 10^{-7} to 10^{-10} M had a significant proliferative effect on the embryo liver cell line WRL68, with a maximum increase of 23.4 % at 10^{-8} M ($p < 0.01$). The addition of anti-G17 caused inhibition of 16.9% on unstimulated

WRL68 ($p<0.01$) and also inhibited the proliferative effect all concentrations of G17 (Figure 3b).

G17 had no proliferative effect on the cell lines PLC/PRF/5, HepG2 and MCA-RH7777 and neither anti-G17 nor PD 135, 158 had any effect on these cell lines (figure 3c-e).

2.8.3 **G17-gly Stimulation**

G17-gly caused a maximum 31.3% rise in cell number at 10^{-9} M ($p < 0.001$) on the positive control AR42J. The addition of anti-G17 inhibited the proliferative effect of G17-gly. In the presence of PD135,158 there was significant inhibition of AR42J cells even in the presence of G17-gly although there was a non-significant trend towards stimulation at G17-gly 10^{-7} M (figure 4a).

G17-gly at concentrations of 10^{-6} to 10^{-11} M had a significant proliferative effect on the embryo liver cell line WRL68, with a maximum increase of 39,7% at 10^{-7} M ($p <0.001$). The addition of anti-G17 caused inhibition of 13.3 % on unstimulated WRL68 cells ($p <0.01$) and also inhibited the proliferative effect of G17-gly. PD135,158 caused a 9.4 % inhibition of unstimulated cells ($p < 0.01$) and also inhibited the proliferative effect of low concentrations of G17-gly but not concentrations > 10^{-8} M G17-gly (figure 4b).

G17–gly 10^{-6} to 10^{-10}M had a significant proliferative effect on the human hepatoma cell line PLC/PRF/5 with a maximum increase of 45.5% at 10^{-10} M ($p <0.001$). The addition of anti-G17 inhibited the proliferative effect of G17-gly. PD135,158 inhibited the proliferative effect of G17-gly at 10^{-11} M but at higher concentrations of G17-gly was unable to inhibit the proliferative response (figure 4c).

G17-gly had no proliferative effect on the cell lines HepG2 and MCA-RH7777 and neither anti-G17 nor PD135, 158 had any effect on these cell lines (fig. 4d-e)

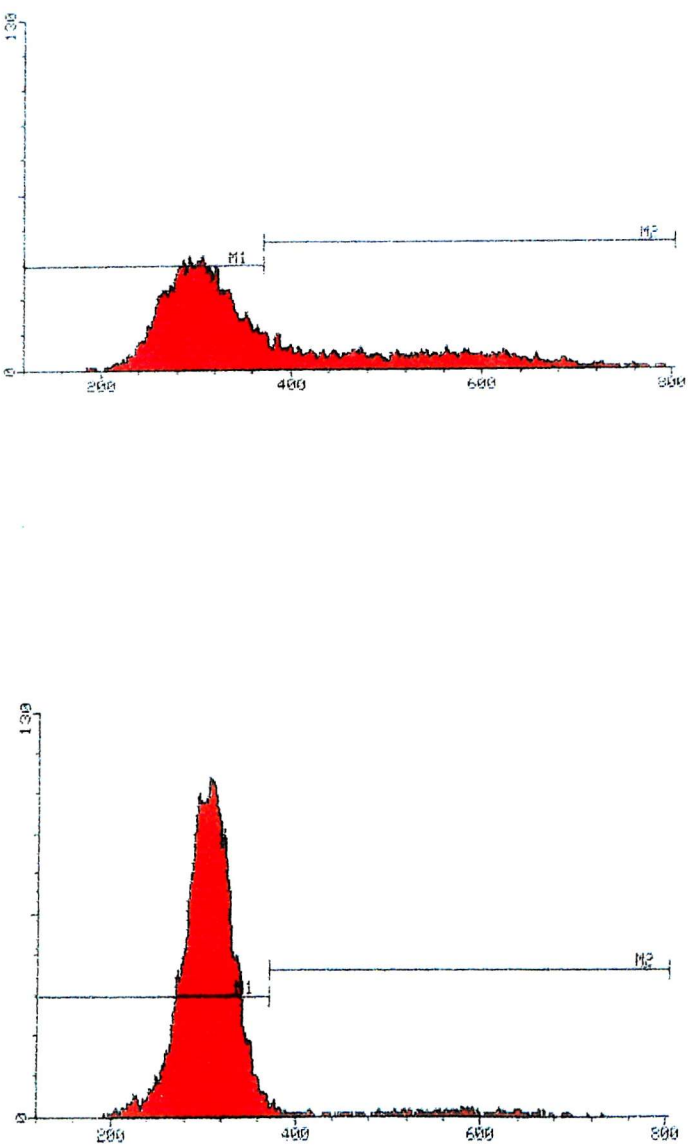


Figure 2
Representative FACS analysis for PLC/PRF/5 cell line 9(a) before synchronisation (b) after synchronisation.
Cells were synchronised using 1mM thymidine and suspended in PBS containing 5µg/ml propidium iodide and 50ng/ml RNAse. Flow cytometry ws performed on a FACScan flow cytometer gating on viable cells.

Figure 3a

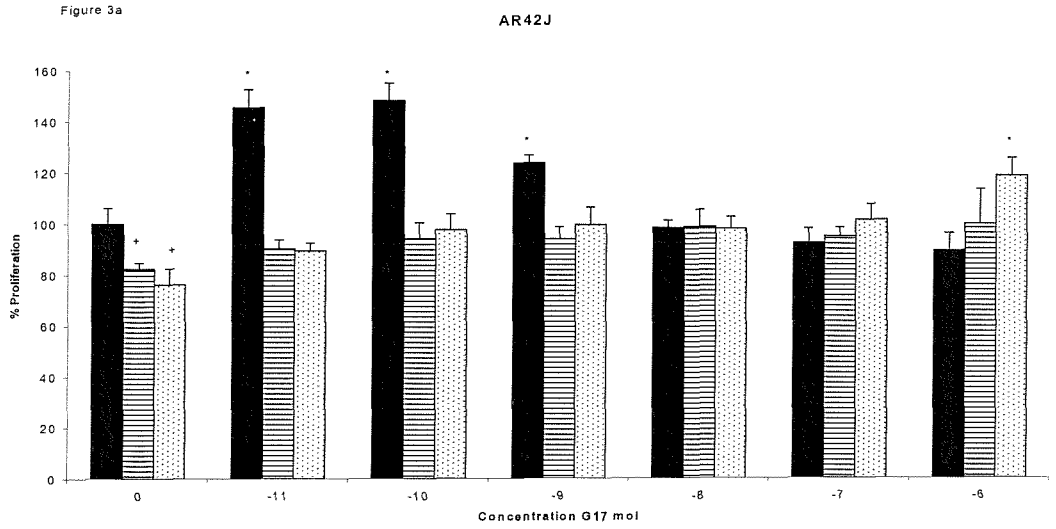


Figure 3c

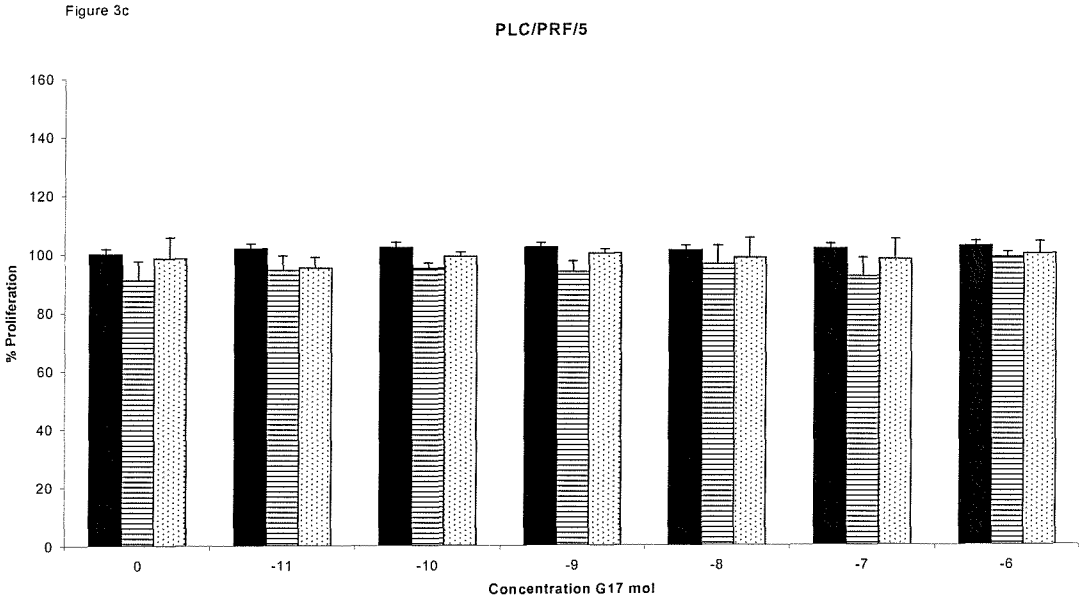


Figure 3b

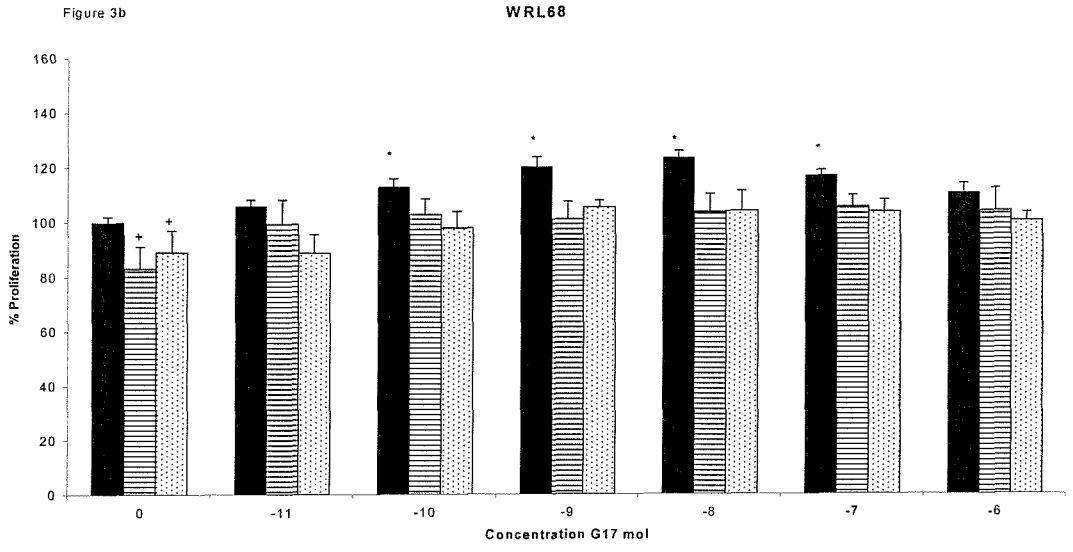


Figure 3d

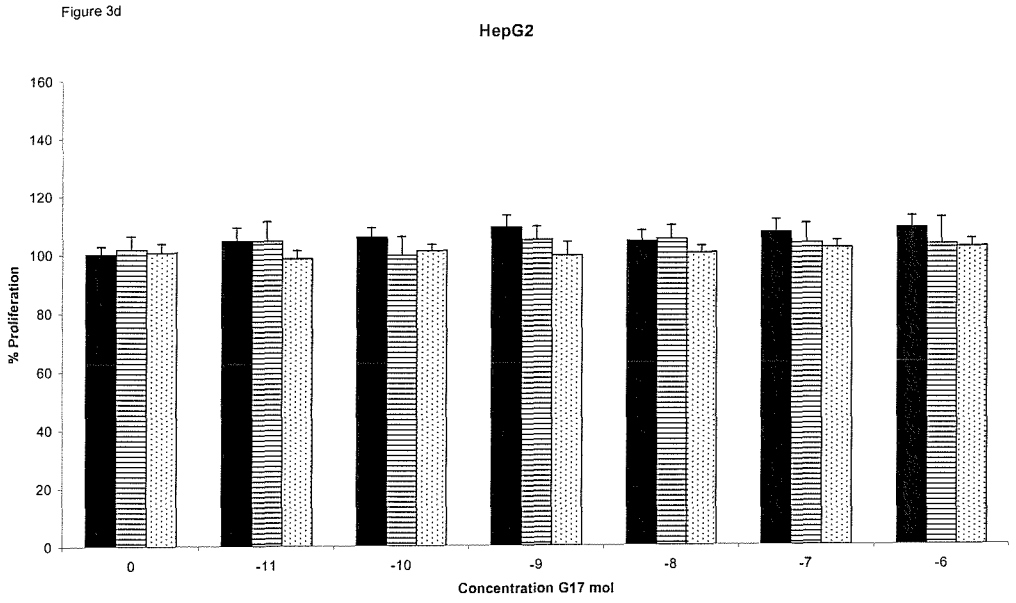


Figure 3e

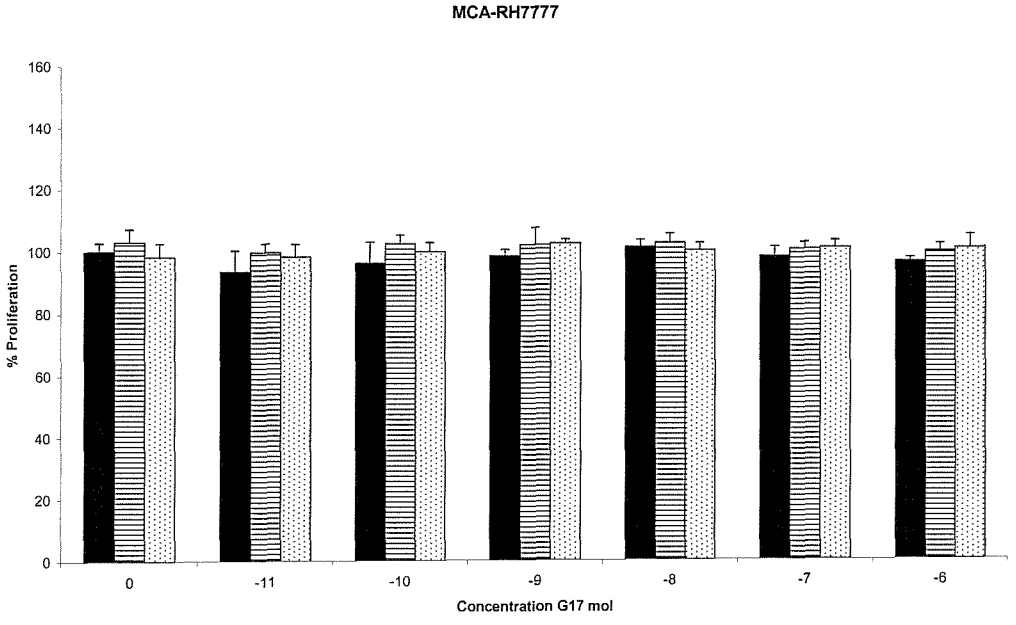


Figure 3

Effect of G17, anti-G17 and PD135,158 on cell proliferation (a) AR42J, (b) WRL68 (c)PLC/PRF/5, (d) HepG2, and (e) MCA-RH7777.
1 x 10^4 cells in 90ul S-Fm (1% media for AR42J cells) were dispensed into 96 well plates. G17 was added at the final concentrations ranging form 10^{-6}-10^{-11} M. Parallel experiments were performed with the addition of anti-G17 and PD135,158. Control experiments used pre-immunised purified rabbit IgG. (+ $p< 0.01$ inhibition; * $p< 0.01$ proliferation)

Figure 4a

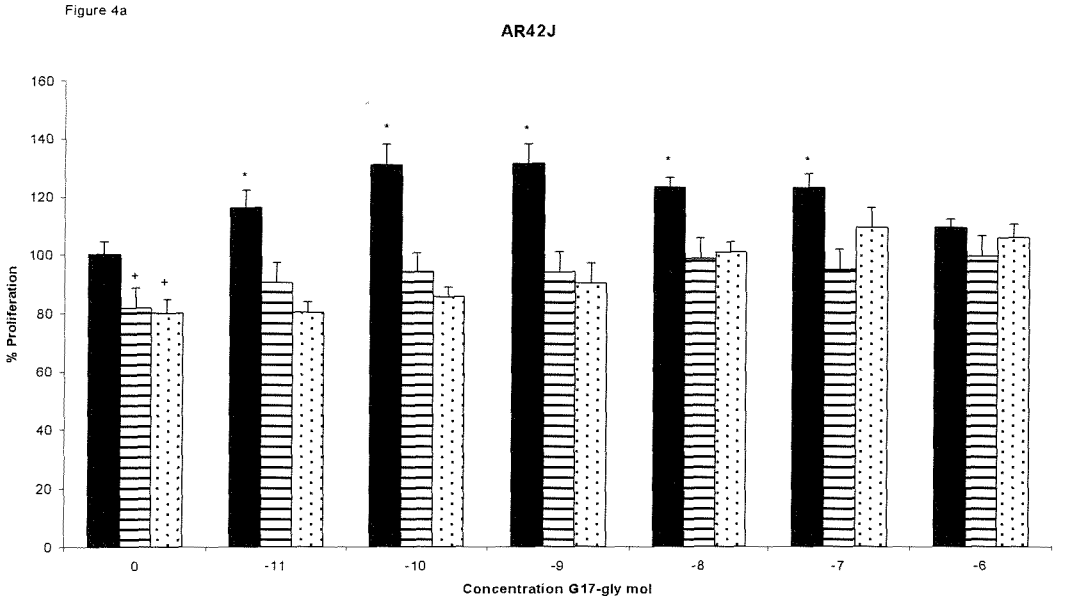
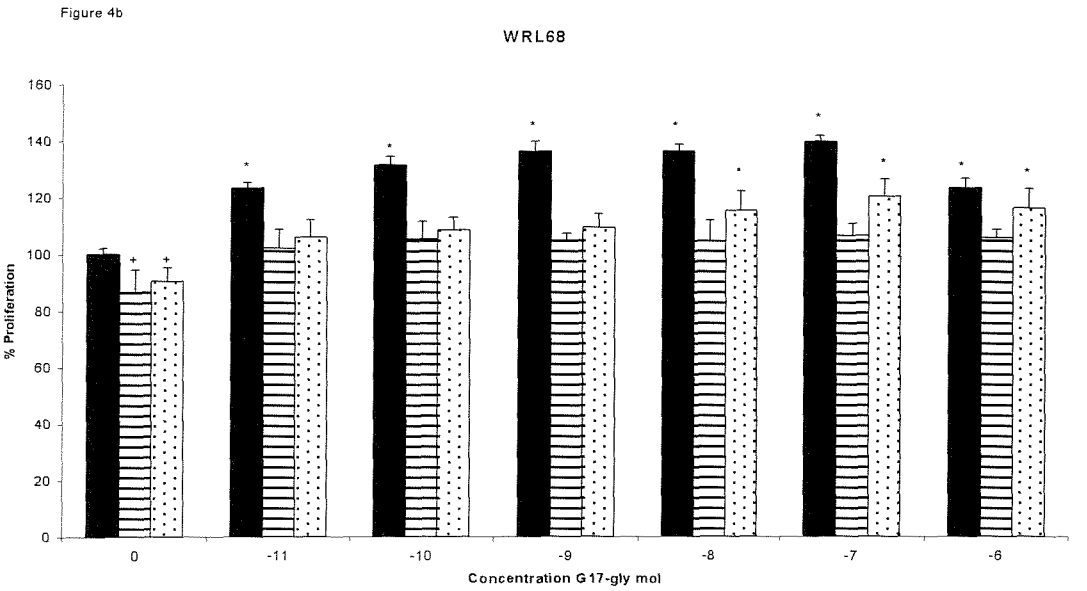


Figure 4b



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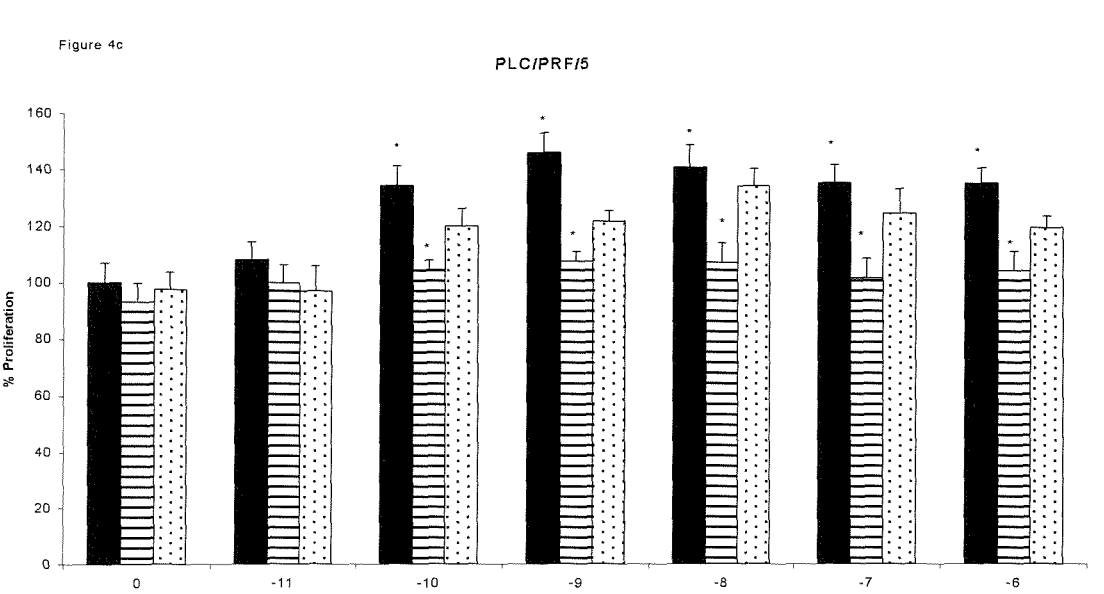


Figure 4d

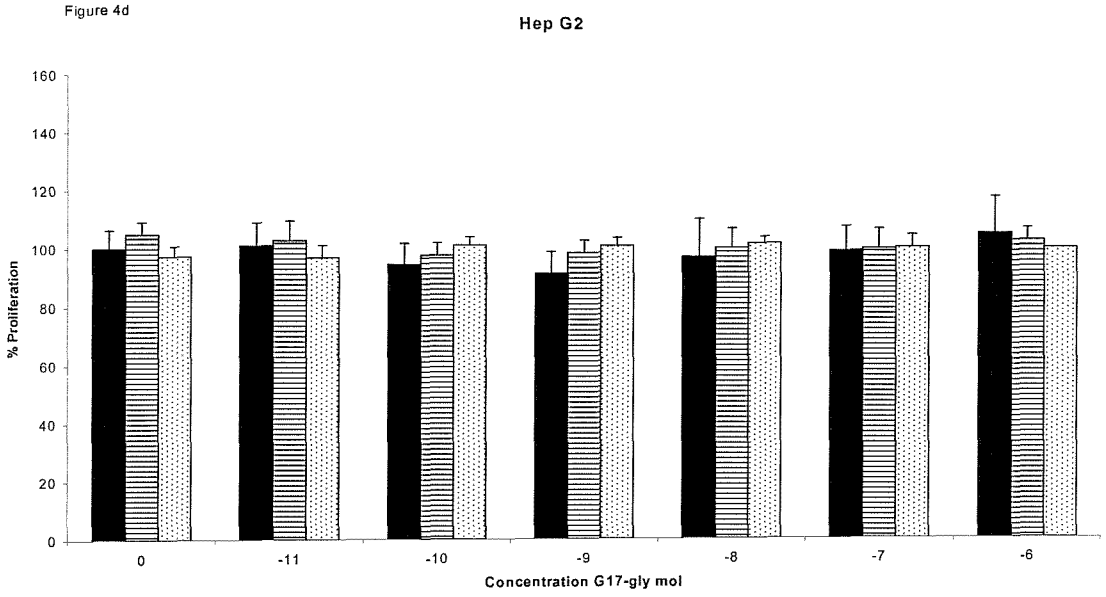


Figure 4e

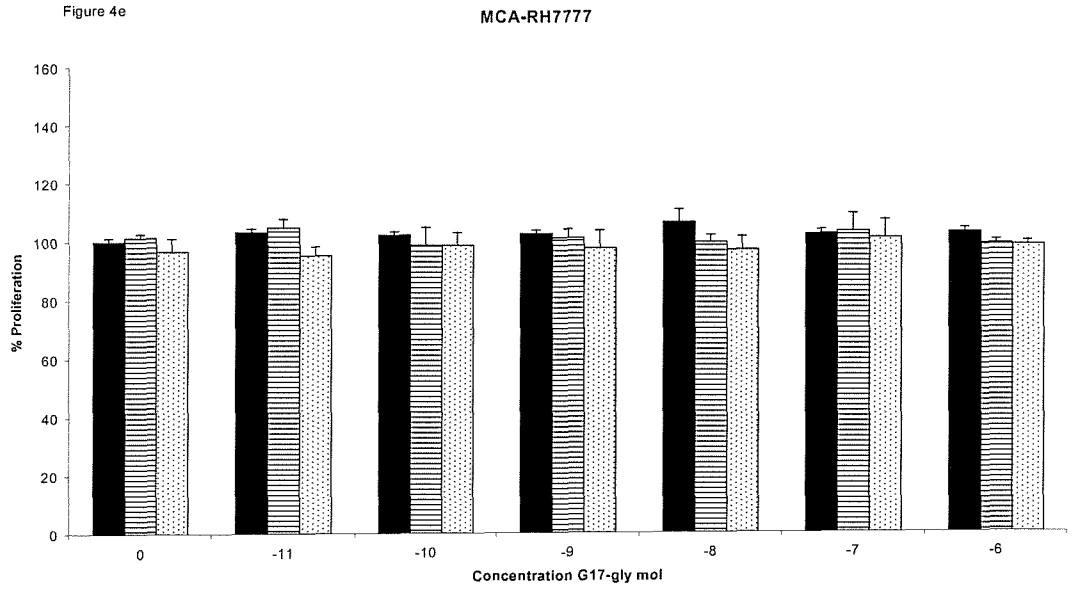


Figure 4
Effect of G17-gly, anti-G17 and PD135,158 on cell proliferation (a) AR42J (b) WRL68 (c)PLC/PRF/5, (d) HepG2, and (e) MCA-RH7777.
1 x 10^4 cells in 90 ul S-Fm (1% media for AR42J cells) were dispensed into 96 well plates. G17-gly was added at final concentrations ranging from 10^{-6}-10^{-11} M. Parallel experiments were performed with the addition of anti-G17 and PD135, 158. Control experiments used pre-immunised purified rabbit IgG.

2.8.4 CCK-B/gastrin Receptor Expression

Immunocytochemical analysis of cytospins identified the CCK-B/gastrin receptor only on the AR42J and WRL68 cell lines (figure 5a-b). Pre-incubation of the antibody with purified receptor antigen quenched the immunostaining.

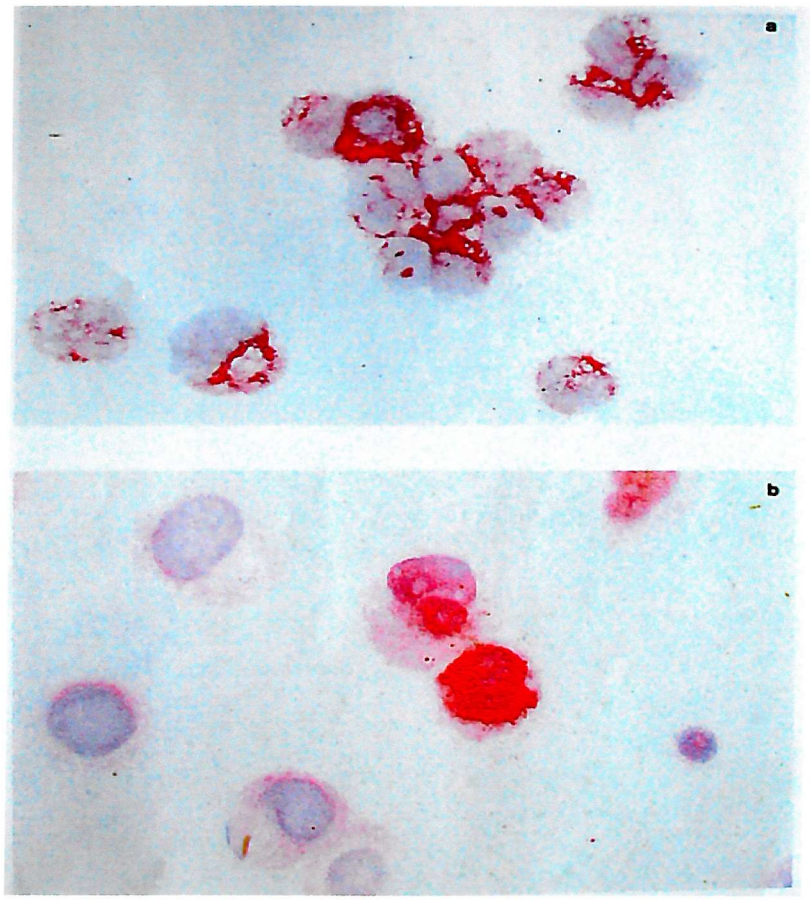


Figure 5
Immunohistochemical identification of the CCK-B/gastrin receptor on cytospins of (a) AR42J pancreatic adenocarcinoma cells (positive control) (b) WRL68 embryonic liver cells.

2.9 Discussion

This study has clearly shown that both G17 and G17-gly are trophic peptides *in vitro*, at concentrations which approximate to physiological levels using the AR42J pancreatic adenocarcinoma cell-line. It is well described that G17 acts via the CCK-B/gastrin receptor, but G17-gly binds to a separate "CCK-C" receptor and that the AR42J cells express both types of receptor (101). This study also showed that high concentrations of G17, well above those found *in vivo*, inhibited AR42J tumour growth. This has been demonstrated previously using this cell line and has been attributed to down-regulation of expression of the CCK-B/gastrin receptor (102). An excess concentration of anti-G17 was used in this study and has been shown previously to effectively compete with CCK-B/gastrin receptors for binding of ^{125} [I] G17 onAR42J cells (72). Studies using the specific CCK-B/gastrin receptor antagonist PD135,158 inhibited the proliferative effect of G17 except at a pharmacological concentration of G17 10^{-6} M. G17-gly did not have a proliferative effect in the presence of PD135,158 although there was a trend towards proliferation at 10^{-7} M G17-gly. This study would therefore suggest that G17 has the greatest proliferative effect on AR42J cells and that this action is mediated by the CCK-B/gastrin receptor. The finding that anti-G17 inhibits unstimulated AR42J suggests that the antibodies may be having their effect by neutralising the autocrine production of G17 and G17-gly. Similarly PD 135,158 inhibits the unstimulated AR42J cells again suggesting that it abrogates the dominant autocrine G17 pathway. Although G17 and G17-gly have not been assayed, the neutralising effect of anti-G17 on autocrine gastrin production has previously been demonstrated on both gastric and colorectal cancer cell lines (78, 103).

FACS analysis of cells grown *in vitro*, showed the variation of growth of tumour cells with respect to different phases of the cell cycle, highlighting the importance of cell synchronisation in increasing the sensitivity of short-term cell proliferation studies (104).

Both G-17 and G17-gly increased the proliferation of the human embryo liver cell line WRL68 which could be inhibited by the addition of anti-G17. The anti-G17 also inhibited the growth of unstimulated cells which implies that the growth of these cells is, at least in part, under the control of autocrine gastrin production PD135,158 was able to inhibit the proliferative effect of G17 but not that of G-gly, again demonstrating that the actions of G17-gly are mediated via a different receptor. The CCK-B/gastrin receptor was identified on WRL68 cells but the receptor binding of G17-gly was not investigated. The proliferative effect of G17 and G17-gly on the embryo liver cell line is not surprising as a number of foetal tissues have been shown to be sensitive to a variety of trophic agents including gastrin (105).

In some animal systems it has been suggested that the effect of gastrin on foetal tissues later becomes suppressed in the adult (106). The subsequent loss of gastrin repression may result in re-expression of embryonic characters together with neoplastic change and this has been described in a transgenic mouse model (107, 56).

Although G17-gly stimulated proliferation of the PLC/PRF/5 cell line at concentrations similar to those found *in vivo*, immunocytochemistry did not reveal the presence of the CCK-B/gastrin receptor. PD135,158 had little effect on the proliferative action of G17-gly and these results again provide indirect support for previous suggestions that G17-gly is acting via a separate receptor. Anti-G17 antibodies did inhibit the proliferative effect of G17-gly stimulated cells, and there

was a reduction that did not however reach statistical significance in unstimulated PLC/PRF/5 cells.

However, G-17 had no effect on PLC/PRF/5 cells, and neither G17 nor G17-gly had any effect on either HepG2 or MCA-RH7777 cell lines. Additionally neither anti-G17 nor PD135,158 had any effect on these cell lines. The lack of effect of gastrin on these immortalised hepatocellular carcinoma cell lines does not preclude a role for gastrin in the biology of these tumours. Similar findings have been described for colorectal carcinoma cell lines, which became less sensitive to gastrin with increasing number of cell passages. When these non-sensitive cell lines were grown in nude mice they recovered their sensitivity to gastrin (71). An alternative theory is that, such cell lines may already be maximally stimulated by the autocrine production of gastrin by the tumour cells (108). The lack of inhibitory effect of anti-G17 militates against this possibility.

In conclusion, this study suggests that there may be a role for gastrin and its precursors in the proliferation of embryonic liver cells and perhaps in the proliferation of some liver tumours.

Chapter 3:

EXPRESSION AND PROCESSING OF GASTRIN IN HEPATOCELLULAR CARCINOMA, FIBROLAMELLAR CARCINOMA AND CHOLANGIOVARCINOMA

Contents

3.1 Introduction

Gastrointestinal cancers have been shown to demonstrate the gastrin autocrine pathway with the precursor forms of gastrin predominating in the tissues examined. Chapter 2 demonstrated that G-NH2 and G17-gly both promoted the growth of a human embryonic liver cell line, and that G17-gly promoted cellular proliferation of a human hepatoma cell line. Animal studies have shown that gastrin may be involved in liver regeneration (95), although other studies have not supported this (96).

The aim of this study was to identify the expression of CCK-B/gastrin receptor (CCK-BR), progastrin, glycine-extended gastrin and amidated gastrin in both normal liver and liver tumours including hepatocellular carcinoma, cholangiocarcinoma and fibrolamellar carcinoma.

3.2 Materials and Methods

3.2.1 Patient Liver Sections

Paraffin sections (5μ thickness) from consecutive unselected patients at the Royal Free Hospital with hepatocellular carcinoma (n = 23 patients), fibrolamellar carcinoma (n = 10), cholangiocarcinoma (n = 5), and normal liver biopsies (n = 10) were assessed. In addition, paraffin sections from a patient with metastatic liver gastrinoma was used as an internal positive control. Negative controls are described below.

3.2.2 Immunohistochemistry

Tissue sections were dewaxed in xylene (Chemicon, UK) for 10 min, dehydrated in 100% alcohol and then rinsed in double distilled water for 5 min. For detection of gastrin receptor and amidated gastrin, slides were microwaved in 0.01M citric acid buffer for 5 min. No pre-treatment was used for progastrin or glycine-

extended gastrin detection. All sections were incubated in 15% acetic acid for 20 min to inhibit endogenous alkaline phosphatase activity. Non-specific binding was blocked by incubating the sections in 10% normal goat serum (DAKO, High Wycombe, UK) in TBS for 15 min. Optimally diluted antibody (see below) was added to each section for 1 hour. Control studies were performed without primary antibody and by preabsorbance of antibody with epitope. Binding was detected using alkaline phosphatase conjugated goat anti-rabbit antibody (Sigma, Poole, UK) at a dilution 1:50 in TBS for 1 hour at room temperature. Sections were washed in TBS for 5 min then visualised using fast red (Sigma) as chromogen incubating at room temperature for 10-20 min. Sections were counterstained in Carrazi's haematoxylin, air dried and mounted in Loctite UV (Loctite, UK) adhesive.

3.2.3 **Antibodies**

CCK-B/gastrin receptor antibody (dilution 1:100) is a rabbit polyclonal antibody raised against the amino-terminal sequence of the human CCK-B/gastrin receptor (Watson et al 1996 (78)).

Progastrin antibody (dilution 1:300) is a rabbit polyclonal antibody raised against human prepro gastrin and reacts with the carboxy-terminal progastrin fragments longer than hexapeptide (Varro et al 1997 (109)).

Glycine-extended gastrin antibody (dilution 1:200) is a rabbit polyclonal antibody raised against human carboxy-terminal glycine extended gastrin and reacts equally with glycine-extended gastrin 17 and glycine-extended gastrin 34 (Varro et al 1997 (109)).

Gastrin antibody (dilution 1:200) is a rabbit polyclonal raised against human carboxy-terminal amidated gastrin 17 and reacts with carboxy-terminal amidated gastrin 34 (DAKO, UK).

3.2.4 Scoring of Immunohistochemistry

All sections were scored independently. Intensity of staining was made in comparison to the positive control gastrinoma tumour cells for each antibody. Immunostaining was scored as follows: strong + + +; moderate + +; weak +; no staining 0. Where only small groups of cells showed immunostaining, rather than the whole tumour sample, this was noted. In addition where there was evidence of expression of receptor or peptide isoforms at the interface of the tumour and normal liver, this was assessed separately.

3.3 Results

3.3.1 **Metastatic Gastrinoma**. (Figures 6a-d) The positive control tissue, liver metastases of gastrinoma, demonstrated a clear distinction between normal liver and gastrinoma tumour, figure 1a-d. There was strong staining of the tumour i.e. + + +, for CCK-BR, proG, G-gly and G-NH2, there was no staining of normal hepatocytes.

3.3.2 **Normal Liver** (Table 1; Figure 7a-d). The sections from patients who had histologically normal liver biopsies showed weak staining for CCK-BR in five (50%) patients, but no expression of proG, G-gly or G-NH2 in any of the samples.

3.3.3 **Hepatocellular carcinoma** (Table 2; Figure 8a-d). Most patients with HCC had significant staining for CCK-BR within the tumour and 65% showed significant staining at the tumour interface. Nine (39%) patients with HCC had significant expression for proG, although two of these patients just showed expression in small tumour foci rather than all tumour cells. A further five (22%) patients showed weak expression for proG. Five (22%) of HCC patients showed weak expression for proG

at the interface. For G-gly, seven (30%) HCC patients demonstrated significant expression, one of these showing focal tumour staining only. Expression (mainly weak) was also seen at the tumour interface in five (22%) of patients. There was no expression of G-NH2 seen in any of the HCC patients.

3.3.4 **Fibrolamellar carcinoma** (Table 3; Figure 9a-d). Nine (90%) patients showed significant staining for CCK-BR and five (50%) had significant staining at the tumour interface. Seven (70%) patients had significant staining for progastrin with one case showing focal staining only. Two other patients showed weak staining within the tumour. There were two patients who showed significant staining at the tumour interface, otherwise there was weak expression seen in six other patients at the tumour interface. Five (50%) patients showed significant staining for G-gly with one patient just showing significant staining in tumour foci. At the tumour interface only one patient showed significant staining. Only one patient showed weak staining for G-NH2 within the tumour otherwise no other staining for G-NH2 was seen.

3.3.5 **Cholangiocarcinoma** (Table 4; Figure 10a-d). Nine (90%) patients with CC had significant staining for CCK-BR and three of these had significant staining at the tumour interface. Six (60%) had significant staining for proG with four showing significant staining and a further patient showing weak staining at the interface. Four (40%) had significant staining for G-gly with two patients showing significant staining at the tumour interface. Three (30%) patients showed weak staining for G-NH2 within the tumour.

No immunostaining was seen in any of the negative controls.

Table 1

Expression of CCK-BR, ProG, G-gly, G-NH2 in normal liver biopsies (n=10)

Antibody	Hepatocyte staining (% of total)
CCK-BR	5/10 (50%) +
ProG	0/10 (0%)
G-gly	0/10 (0%)
G-NH2	0/10 (0%)

Staining Score: 0 = none; + = mild; ++ = moderate; +++ = intense

Table 2.

Expression of CCK-BR, ProG, G-gly and G-NH2 in hepatocellular carcinoma (n=23)

Antibody	Tumour staining (% of total)	Tumour interface staining (% of total)
CCK-BR	21/23 (91%) ++	15/23 (65%) ++
ProG	*9/23 (39%) ++ [*2 focal] 5/23 (22%) +	3/9 (13%) + 2/5 (9%) +
G-gly	*7/23 (30%) ++ [* 1 focal] 4/23 (17%) +	3/7 (13%) +; 1/7 (4%) ++ 1/4 (4%) +
G-NH2	0/23 (0%)	0 (0%)

Table 3.

Expression of CCK-BR, ProG, G-gly and G-NH2 in fibrolamellar carcinoma (n=10)

Antibody	Tumour staining (% of total)	Tumour interface staining (% of total)
CCK-BR	9/10 (90%) ++	5/10 (50%) ++
ProG	*7/10 (70%) ++ [*1 focal] 2/10 (20%) +	5/7 (50%) +; 1/7 (10%) ++ 1/2 (10%) +; 1/2 (10%) ++
G-gly	*5/10 (50%) ++ [*1focal] 2/10 (20%) +	4/5 (40%) +; 1/5 (10%) + 2/2 (20%) +
G-NH2	1/10 (10%) +	0 (0%)

Table 4

Expression of CCK-BR, ProG, G-gly and G-NH2 in cholangiocarcinoma (n=10)

Antibody	Tumour staining (% of total)	Tumour interface staining (% of total)
CCK-BR	9/10 (90%) ++	4/9 (40%) ++; 1/9 (10%)+
ProG	*6/10 (60%) ++ [*2 focal] 3/10 (30%) +	4/7 (40%) ++; 1/7 (10%) + 1/10 (10%) ++
Gly-G	*4/10 (40%) ++ [*1 focal] 3/10 (30%) +	2/10 (20%) ++ 1/10 (10%) +
G-NH2	3/10 (30%) +	0 (0%)

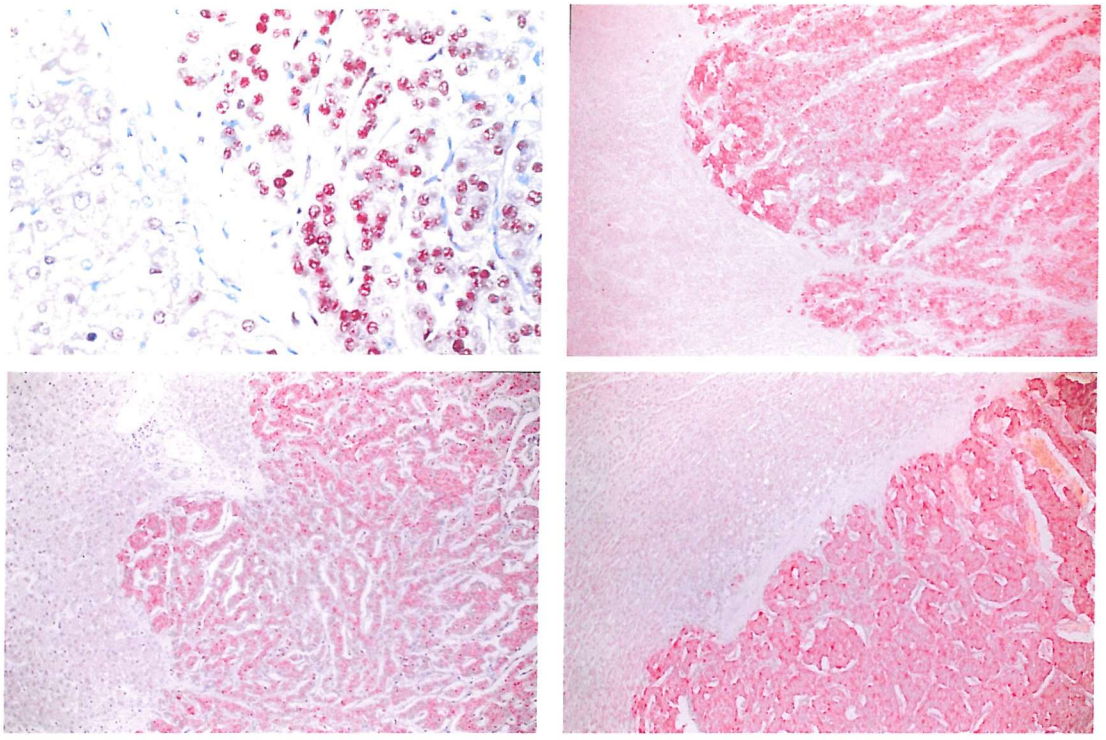


Fig. 6. Liver section demonstrating metastatic gastrinoma with surrounding normal liver. The metastatic gastrinoma was used as a positive control for the expression of (a) CCK-BR, (b) ProG, (c) G-gly and (d) G-NH2. Magnification A-D: ×100 (alkaline phosphatase methodology).

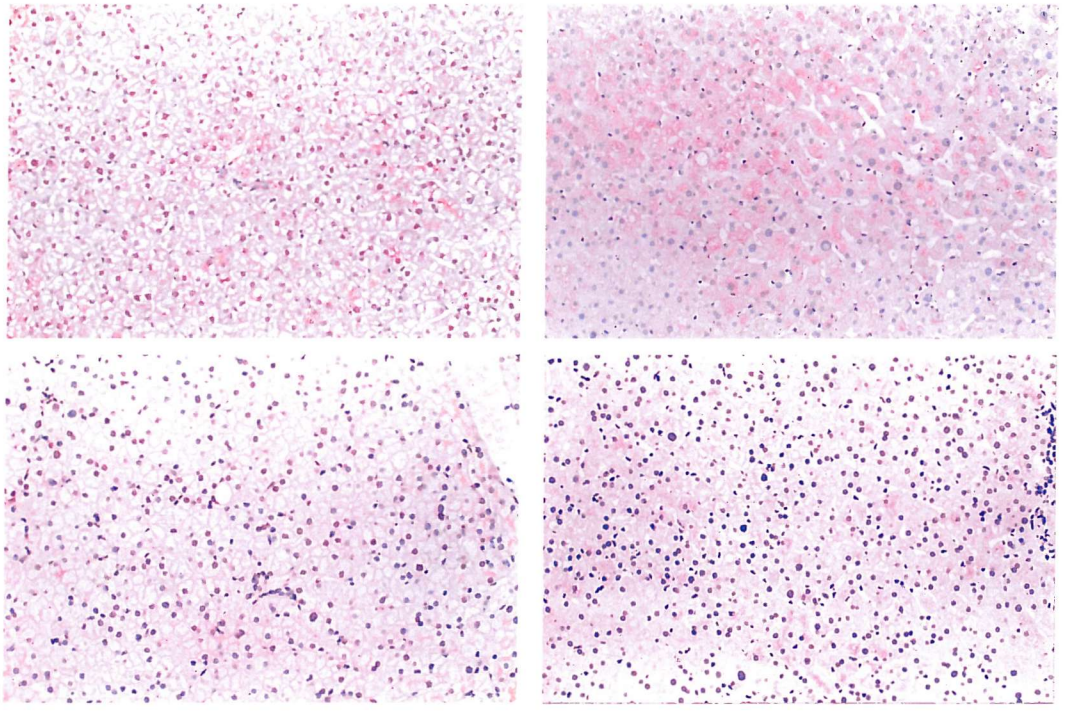


Fig. 7. Normal liver biopsies demonstrating minimal expression of CCK-BR (a), and no expression of (b) ProG, (c) G-gly and (d) G-NH2. Magnification A-D: ×200 (alkaline phosphatase red methodology).

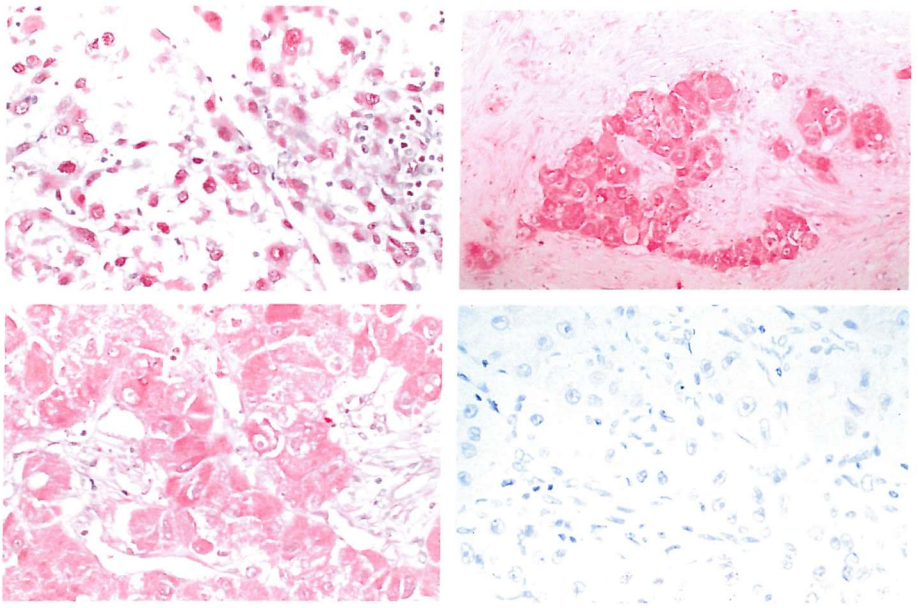


Fig. 8. Hepatocellular carcinoma demonstrating expression of (a) CCK-BR, (b) ProG, (c) G-gly but no expression of (d) G-NH2. Magnification: ×400 (alkaline phosphatase red methodology).

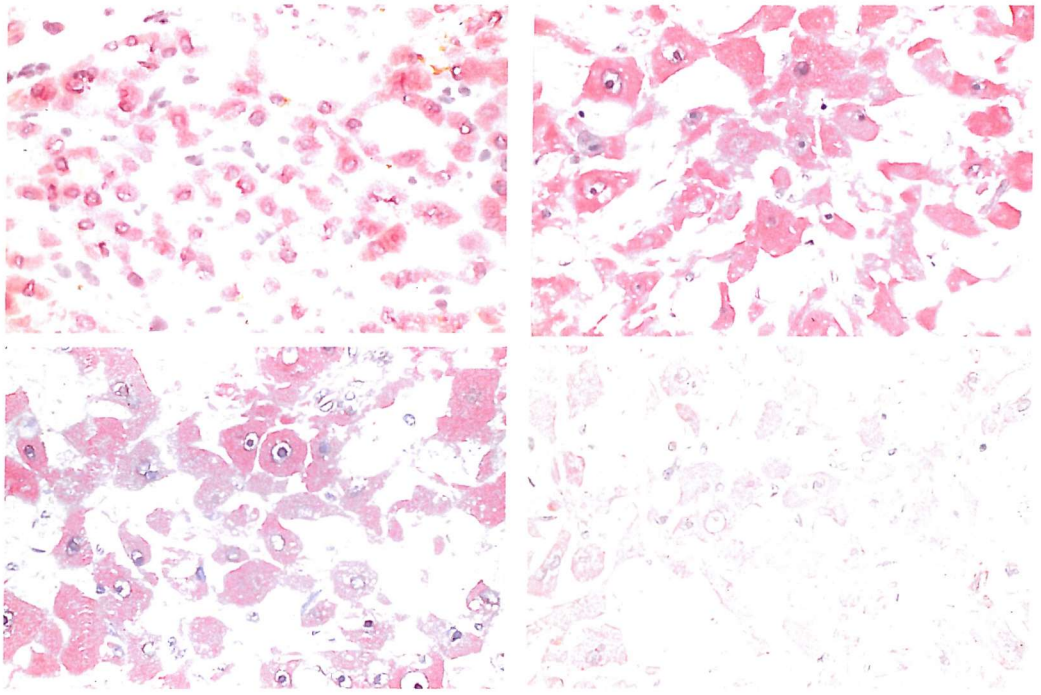


Fig. 9. Fibrolamellar carcinoma demonstrating expression of (a) CCK-BR, (b) ProG, (c) G-gly with equivocal staining for (d) G-NH2. Magnification: ×400 (alkaline phosphatase red methodology).

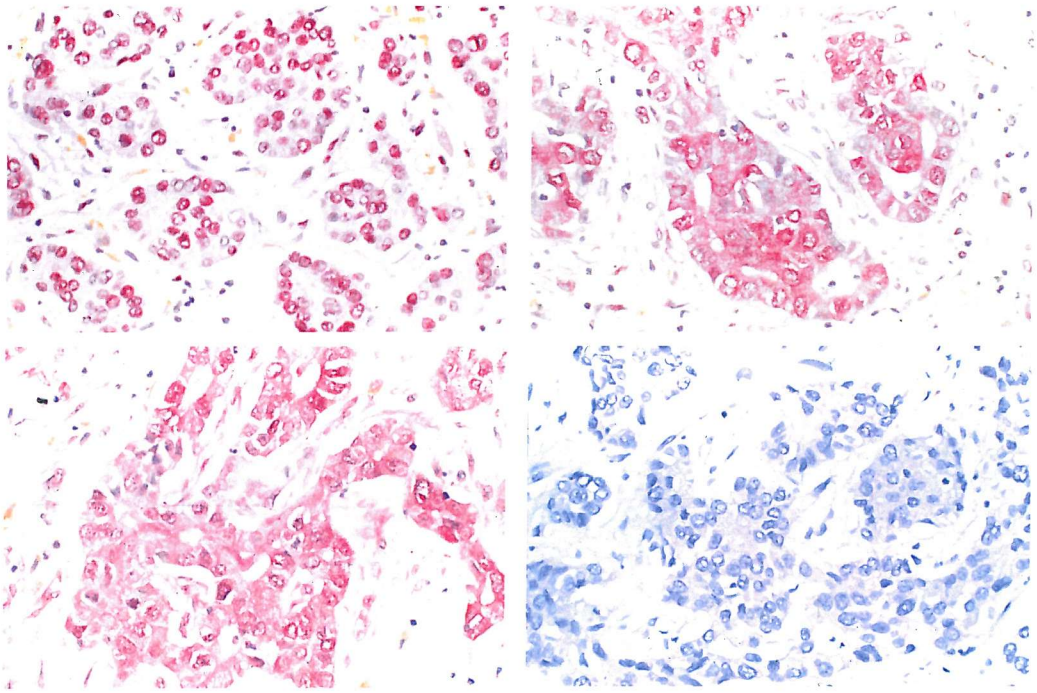


Fig. 10. Cholangiocarcinoma demonstrating expression of (a) CCK-BR, (b) ProG, (c) G-gly but no expression of (d) G-NH2. Magnification×400 (alkaline phosphatase red methodology).

3.4 Discussion

This study has demonstrated that gastrin receptor as well as precursor forms of gastrin principally the progastrin and glycine-extended forms, are expressed in liver tumours. These immunohistochemical findings are validated by the preabsorbtion studies and the lack of staining seen in the normal liver. The gastrinoma specimen demonstrated very intense immunocytochemical staining for CCK-BR and gastrin isoforms. The immunostaining results in other tumours and normal sections were scored by comparison with the gastrinoma and are therefore a conservative interpretation of receptor and peptide expression.

Some of the carcinoma sections show only foci of tumour staining for gastrin isoforms. This is not an unusual finding and may represent clonal or tumour divergence of some tumour cells (110).

The presence of gastrin receptor and gastrin isoforms have previously been demonstrated in other gastrointestinal carcinomas and non-gastrointestinal cancers. Such tumours are theoretically capable of responding not only to circulating "endocrine" forms of gastrin (including hypergastrinaemia secondary to pharmacological intervention), but also the autocrine production of gastrins by tumour tissue itself. There is now clear evidence for the importance of the latter pathway in gastrointestinal cancers (78, 111) as well as pancreatic cancer (112).

There was expression of the CCK-BR in most HCC, FLC and CC sections. Expression of the receptor has been found in many non-gastrointestinal tumours including leukaemias (113) and astrocytic tumours (114) in addition to those tumours mentioned above. The biological significance of tumour expression of receptor in so many diverse tumours remains to be determined. The situation is further complicated by the fact that G-gly binds to a separate CCK- "C" receptor, which remains to be

characterised. In the light of the demonstration that at physiological concentrations G-gly may have a greater trophic effect on hepatocytes than G-NH2, determination of CCK- "C" receptor expression in the future will be important. It is conceivable that proG may also have some trophic actions in its own right.

The expression of gastrin and precursor forms in liver cancers could be due to either liver uptake of these peptides or autocrine production of peptide by the tumour. In view of the evidence suggesting that the liver is unable to metabolise exogenous gastrin greater than 17 peptides in length (92), the most likely explanation is that these tumours are producing isoforms of gastrin, which may be acting in an autocrine fashion. Recently, Hollande et al suggested that in addition to the "classical" extracellular autocrine loop, there is also an intracellular, intracrine loop, where progastrin-derived peptides that are not released externally, are bound to intracellular receptors and stimulate cellular proliferation (115).

The expression of gastrin isoforms in different tumours including liver, is dependent to a large degree on the presence of a regulated secretory pathway. The tumour with the greatest expression of progastrin and glycine-extended gastrin is the FLC and this is perhaps not unexpected as these tumours often show neuroendocrine differentiation (116, 117).

In a transgenic mouse model containing a human gastrin minigene, the livers of these mice expressed abundant gastrin mRNA and human proG but were unable to process the proG to G-NH2 (56). There is some similarity to the liver tumour samples that were assessed in this study being able to express precursor forms of gastrin but unable to express the "mature" amidated gastrin. In normal liver biopsies there was no expression of progastrin derived peptides and one might conclude that non-neoplastic hepatocytes do not have activation of the gastrin gene.

In other studies, two separate transgenic mouse lines were formed using two fused genes. A Simian virus 40 large tumour antigen (SV40 Tag) gene was fused to a 1.5kb 5' flanking region of the gastrin gene; in another model the SV40 Tag gene was fused to a 10.5kb mid-region of the gastrin gene. The former model developed bile duct tumours and hepatocellular dysplasia, whereas the latter developed biliary duct dysplasia and focal hepatocellular carcinoma (107). This again suggests a relationship between gastrin and liver tumours consistent with our findings.

In summary, this study has shown that most liver tumours express the CCK-B/gastrin receptor and a significant proportion of liver tumours demonstrate expression of proG and G-gly. The tumours do not appear to be able to process progastrin to the mature amidated gastrin. Evidence from cell culture studies and comparison with human gastrointestinal cancers, suggests that precursor forms of gastrin may have a proliferative effect on neoplastic hepatocytes. Liver cancers have a dreadful prognosis and the expression of gastrin receptor and gastrin isoforms is worth investigating further, as it may not only lead to improved understanding of the pathophysiology of these tumours but may also allow the development of therapeutic opportunities.

Chapter 4:

EXPRESSION AND PROCESSING OF GASTRIN IN PANCREATIC ADENOCARCINOMA

Contents

4.1 **Introduction**

Cell-culture studies and animal models suggest that as for gastrointestinal and hepatocellular cancers, pancreatic adenocarcinoma cells not only have the ability to respond to circulating forms of gastrin but also respond to the autocrine production of gastrin and its precursors (112).

Gastrin promotes the growth of a rat pancreatic cancer cell line, AR24J, in vitro (118). The growth of pancreatic xenograft in nude mice is promoted by the addition of pentagastrin, and this effect is blocked by the addition a CCK-B receptor antagonist or an antigastrin antibody, while CCK-A receptor antagonists have no effect (88, 119).

Glycine-extended gastrin, like amidated gastrin, can promote the growth of the rat pancreatic cancer cell line AR42J increasing the rate of [^{3}H]-thymidine incorporation in a dose dependant fashion. CCK-B receptor antagonists block the effects of amidated gastrin, but do not alter the effects of G17-gly (120). It is presumably acting through a different receptor, as previously discussed. AR42J cells also secrete G17-gly in an autocrine pathway for stimulation and neutralisation of G17-gly successfully inhibits the growth of these cells (112).

In a recent study of pancreatic cancer resection specimens, using specific radioimmunoassays, amidated gastrin was found in 14/19 of pancreatic cancer specimens and in 12/15 resection margin specimens. Glycine-extended gastrin was found in few tumours, but progastrin was found in all 19. These investigators also found CCK-B receptor mRNA in pancreatic cancer tissue, tumour resection margins and normal pancreatic tissue by RT PCR (121). There is a divergence of opinion about the expression of the CCK-B receptor in pancreatic tumours, because some studies show a higher expression of CCK-B receptor in pancreatic cancer cell lines over

normal pancreatic tissue (122), but other others find a high prevalence of the CCK-B receptor in the normal pancreas (123). The autocrine stimulation of these cells may be mediated through receptors other than CCK-B/gastrin.

The aim of this study was to identify the expression of CCK-B/gastrin receptor, progastrin, glycine-extended gastrin and amidated gastrin in both normal pancreas and pancreatic adenocarcinoma.

4.2 Materials and Methods

4.2.1 Patient Pancreas Sections

Paraffin sections (5μ thickness) from consecutive unselected patients at the Royal Free Hospital with pancreatic carcinoma (n = 22 patients) and normal pancreatic biopsies (n = 10) were assessed.

4.2.2 Immunohistochemistry

As per chapter 3.2.2

4.2.3 Antibodies

As per chapter 3.2.3

CCK-B gastrin receptor antibody (dilution 1:100)

Progastrin antibody (dilution 1:300)

Glycine-extended gastrin antibody (dilution 1:200)

Gastrin antibody (dilution 1:200)

4.2.4 Scoring of Immunohistochemistry

All sections were scored independently by two experienced histopathologists. The inter-observer variation and intra-observer variation was <10%. Where there was variation in score the section was jointly reviewed and score agreed. Immunostaining was scored as follows:

strong +++; moderate ++; weak +; no staining 0.

4.3 Results

The normal pancreas showed no expression of CCK-B/gastrin receptor, progastrin, glycine-extended gastrin or amidated gastrin except for occasional cells in the islets (Table 5 & figure 11). The pancreatic cancer cells showed definite expression (that is greater than ++) of the CCK-B/gastrin receptor in 96%, progastrin in 92%, glycine-extended gastrin in 54%, and amidated gastrin in 23% of cancer sections (Table 6 & figure 12). Pancreatic cancer was often seen in the region of nerve fibres (figure 13).

TABLE 5

Expression of CCK-B/gastrin receptor, progastrin, glycine-extended gastrin and amidated gastrin in normal pancreas (*n*=10)

Antibody	Intensity of Staining in Normal Pancreas
CCK-B/gastrin Receptor	occasional cells in islets/ no staining of acinar cells
Progastrin	occasional cells in islets/ no staining of acinar cells
Glycine-extended gastrin	occasional cells in islets/ no staining of acinar cells
Amidated gastrin	occasional cells in islets/ no staining of acinar cells

TABLE 6

Expression of CCK-B/gastrin receptor, progastrin, glycine-extended gastrin and amidated gastrin in pancreatic cancer (*n*=22)

Antibody	Intensity of Tumour Staining
CCK-B/gastrin Receptor	3/22 +++ 18/22 ++ 1/22 +
Progastrin	6/22 +++ 14/22 ++ 1/22 + 1/22 0
Glycine-extended gastrin	12/22 ++ 6/22 + 4/22 0
Amidated gastrin	5/22 ++ 2/22 + 15/22 0

Staining score: 0=none; +=mild; ++=moderate; +++=intense

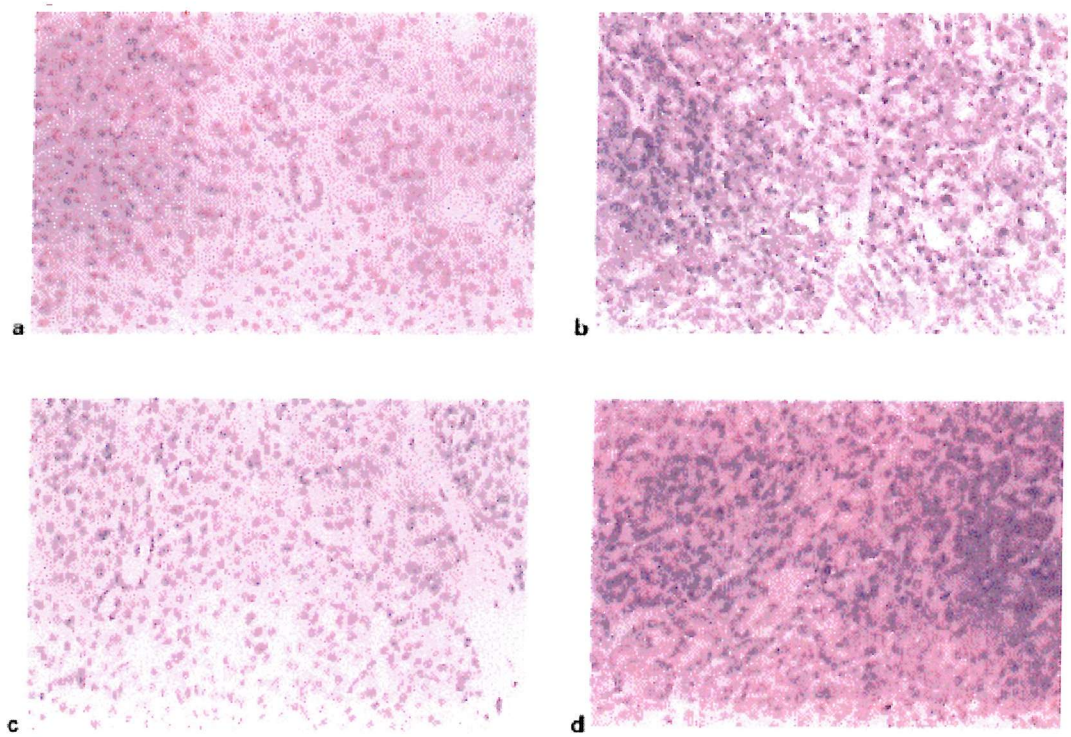


Fig. 11 Alkaline phosphatase red immunohistochemistry of normal pancreas demonstrating no expression of **a** CCK-B/gastrin receptor, **b** progastrin, **c** glycine-extended gastrin and **d** amidated gastrin. (Original magnification ×200)

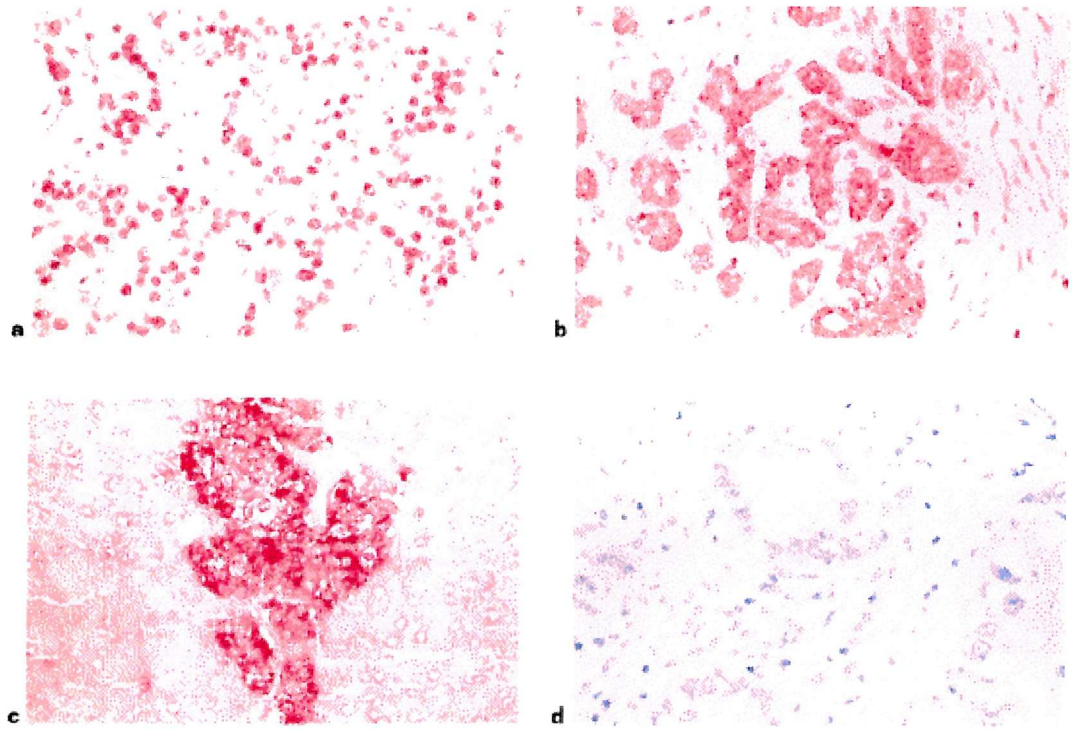


Fig. 12 Alkaline phosphatase red immunohistochemistry of pancreatic cancer demonstrating expression of **a** CCK-B/gastrin receptor, **b** progastrin and **c** glycine-extended gastrin but no expression of amidated gastrin (**d**). (Original magnification ×200)

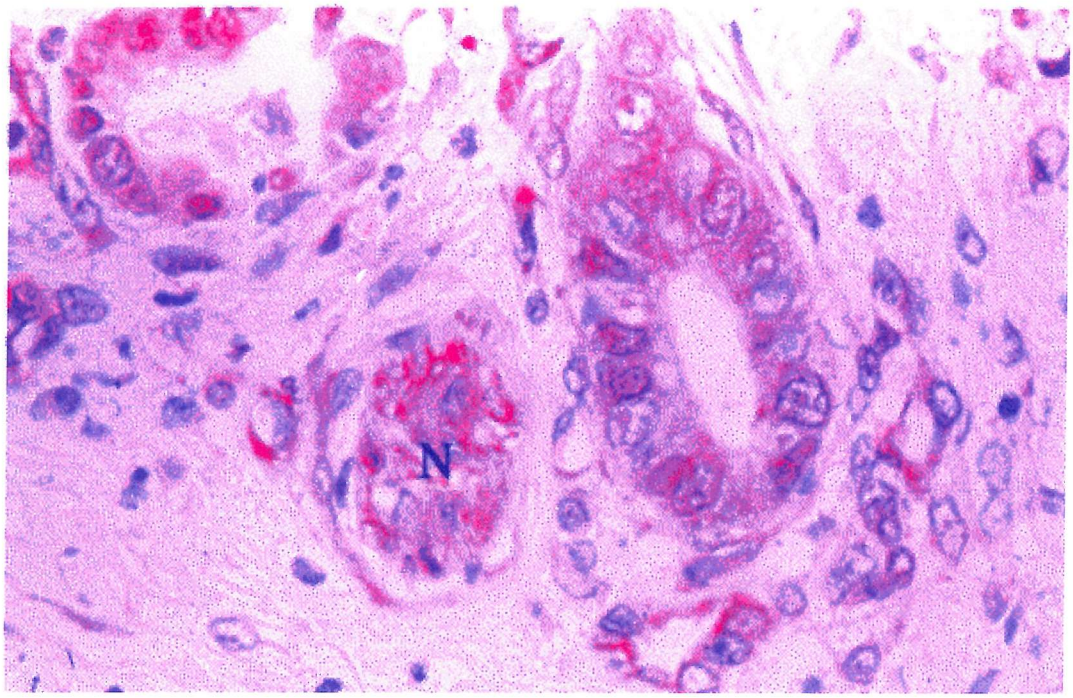


Fig. 13 Alkaline phosphatase red immunohistochemistry of pancreatic cancer stained or progastrin shown in close proximity to a nerve (N). The nerve fibres themselves show some expression of the peptide. (Original magnification × 300)

4.4 Discussion

This study has demonstrated that pancreatic adenocarcinoma expresses the CCK-B/gastrin receptor as well as precursor forms of gastrin, especially the progastrin and glycine extended forms. This is similar to the findings for other cancers including gastric, colon and hepatocellular carcinomas as previously discussed.

Gastrin is known to be transiently expressed in the foetal pancreas; it is associated with pancreatic growth and differentiation but does not appear to be expressed in the normal adult pancreas (124, 125). Many tumours have an onco-foetal expression of peptides and this may apply in relation to gastrin and the pancreas.

Gastrin has been shown to promote the growth of pancreatic adenocarcinoma cell lines *in vitro* (119), and also *in vivo* in the rat using azaserine-induction of pancreatic adenocarcinoma (126) as well as in mouse xenograft models (88). Anti-gastrin agents including CCK-B/gastrin receptor antagonists have been shown to inhibit tumour proliferation in both the *in vitro* and *in vivo* models (88, 127). There are currently no "CCK-C" receptor antagonists, but anti-gastrin antibodies which bind both amidated and glycine-extended forms of gastrin will inhibit the proliferative effect of glycine-extended gastrin in pancreatic cancer (112, 127).

There is some controversy in the literature whether the CCK-B/gastrin receptor is expressed in normal human pancreatic tissue and pancreatic adenocarcinoma (122, 123). This study has shown that only a minority of islet cells in the normal pancreas and no acinar cells expressed the CCK-B/gastrin receptor and gastrin forms. This suggests that in neoplastic pancreatic tissue there is a switch-on of gastrin gene expression, which would fit with the hypothesis of onco-foetal expression of gastrin.

It has been previously established that gastrin stimulates the growth of pancreatic cancer by an autocrine mechanism (112, 127). There is constitutive release of amidated gastrin and its precursor forms, which feed back not only to its own cell but also to neighbouring cells thus having paracrine effect. Amidated gastrin binds to the CCK-B/gastrin receptor but the precursor forms bind to an as yet uncharacterised "CCK-C" receptor. As already mentioned, these precursor forms of gastrin have also been shown to be trophic in their own right and may be even more potent than amidated gastrin.

Transgenic mouse models provide supporting evidence for the trophic effects of gastrin at the gene level. In the previously mentioned mouse model which involved the fusion of a SV40 Tag gene to a 1.5kb 5' flanking region of the gastrin gene or the SV40 Tag gene fused to a 10.5kb mid-region of the gastrin gene; both resulted in the mice developing pancreatic tumours (107).

Although most pancreatic cancer specimens assessed expressed progastrin, only 54% definitely (that is greater than ++ staining intensity) expressed glycine-extended gastrin and 23% amidated gastrin. This is almost certainly a conservative estimate due to the limited sensitivity of immunohistochemistry. However, the relative lack of mature amidated gastrin is probably, as per liver cancers (chapter 3), a reflection of the cellular machinery within tumours i.e. the cancer cell often does not have the capability, associated with the enzymatic pathway, for fully processing the precursor forms of gastrin.

When patients with pancreatic cancer were compared to normal subjects, Smith et al reported a two-fold increase in fasting serum gastrin and ten-fold greater receptor binding capacity in pancreatic cancer patients (128). They postulated that the

aggressive behaviour of pancreatic adenocarcinoma might be attributable to the autocrine production of gastrin and to the presence of its growth-related receptor.

It is recognised that pancreatic cancer cells are often found in the proximity of nerves. A number of studies have shown that pancreatic cancer infiltrates local nerves (129). Dang et al found that pancreatic cancer invaded 73% of pancreatic nerve fibres and also metastasised along nerve fibres in 60% of cases (130). The proposed explanations for this include mechanical extension along planes of least resistance such as the perineural space (131) and, more recently, that specific factors including peptides enhance the intimate relationship of cancer cells and nerves (132). This latter explanation has been proposed in relationship to transforming growth factor and epidermal growth factor as well as their receptors. It is conceivable that this hypothesis could also apply to gastrin, as it is also a neuropeptide.

Pancreatic cancer has a dreadful prognosis and none of the currently available therapies offers a significant survival advantage. New therapeutic strategies need to be developed and this study supports the concept that targeting gastrin and its receptor may provide a novel treatment option.

Chapter 5:

DEMONSTRATION OF NEW SITES OF EXPRESSION OF THE CCK-B/GASTRIN RECEPTOR IN PANCREATIC ACINAR AR42J CELLS USING IMMUNOELECTRON MICROSCOPY

Contents

5.1 Introduction

In terms of ligand binding to the CCK-B receptor it has classically been considered that peptide hormones bind to surface membrane receptors with the resulting formation of secondary messengers regulating cell function (133). Previous studies, using immunohistochemistry, presented in this thesis, have suggested that there may be expression of the CCK-B receptor both within the cytoplasm and nucleus of tumour cells and others have demonstrated cytoplasmic expression of the receptor (134). The aim of this study is to demonstrate the specificity of a CCK-B/gastrin receptor antibody, αCCKBR-Ser, and also demonstrate cytoplasmic and nuclear expression of the CCK-B/gastrin receptor using two independent methods, immunoelectron microscopy (immunoEM) and Western blotting.

(The work on raising the CCK-B/gastrin receptor antibody, the specificity studies using inhibition ELISA assays, radioligand binding inhibition studies and immunofluorescence binding studies were performed by research collaborators and are therefore presented in Appendix 1. Similarly the Western blotting was also performed by collaborators and is also presented in Appendix 1. In view of my input into designing these studies and as the results are integral to the demonstration of expression of the CCK-B/gastrin receptor, I will include the interpretation of these additional studies within the discussion of this chapter.)

5.2 Materials and Methods

5.2.1 Cell Culture

AR42J cells, passage nos. 16-18 (European Collection of Animal Cell Cultures, Porton Down, UK) were derived from a rat pancreatic adenocarcinoma (16). Cells were cultured in RPMI-1640 medium containing 10% FCS and 2mM glutamine. All cells were maintained at 37°C in 5% CO_2 in air at 100% humidity; they were grown to 80% confluence in T75 flasks (Falcon, London, UK) and passaged following 0.02% EDTA to bring adherent cells into suspension.

5.2.2 CCK-B/gastrin Receptor Antibody (α-CCKBR-Ser Antibody)

See appendix 1.2.1

5.2.3 Specificity studies for α-CCKBR-Ser Antibody

Appx 1.2.2 **Inhibition ELISA Studies**

Appx 1.2.3 **Radioligand binding inhibition studies**

Appx 1.2.4 **Immunofluorescence detection of binding**

5.2.4 Immunoelectron Microscopy

Cells were incubated with CCK-B/gastrin receptor antibody, α-CCKBR-Ser antibody, 0.6mcg/ml, for 1h at room temperature. The cells were fixed in 1% glutaraldehyde for 1h. The cell suspension was spun twice at 2000rpm for 2 min and then the cell pellet resuspended in phosphate buffered saline. The cell pellet was infiltrated with LRWhite resin (Sigma, Poole, Dorset). Ultrathin sections of 70-90μm were cut and

placed on Pioloform coated nickel grids. Grids were incubated with 5% normal goat serum (Dako, High Wycombe, UK) in 0.1% bovine serum albumin (BSA) (Sigma, Poole, Dorset) at room temperature for 30 min. Grids were rinsed in PBS (5 x 5min) then incubated with biotin-conjugated goat anti-rabbit antibody (Dako, High Wycombe, UK), diluted 1:50 in 1% BSA for 1h at room temperature. Grids were rinsed in PBS (5 x 5min) then incubated with goat anti-biotin antibody conjugated to 10nm gold for 1h at room temperature. After a final PBS wash the grids were counterstained in saturated aqueous uranyl acetate for 3min, and Reynolds lead citrate for 3min. Control experiments were performed (i) using preabsorbed α-CCKBR-Ser antibody (ii) substituting labelled serine spacer epitope instead of α-CCKBR-Ser and (iii) without primary antibody. Gold particles on the cell membrane, in the cytoplasm, on the nuclear membrane and within the nucleus were counted. Twenty-five cells/grid were counted by a blinded observer.

5.2.5 **Western Blotting**

See appendix 1.2.5

5.3 **Results**

The peptide inhibition ELISA studies, radioligand binding inhibition studies and immunofluorescence studies all confirmed specific binding of the anti-CCK-B/gastrin receptor antibody (see appendix 1 results). The Western blots detected the expression of receptor protein in both extra-nuclear and nuclear extracts (see appendix 1 results).

ImmunoEM studies using α-CCKBR-Ser antibody showed that after one hour incubation the distribution of immunogold labelled CCK-B receptor antibody was 12%

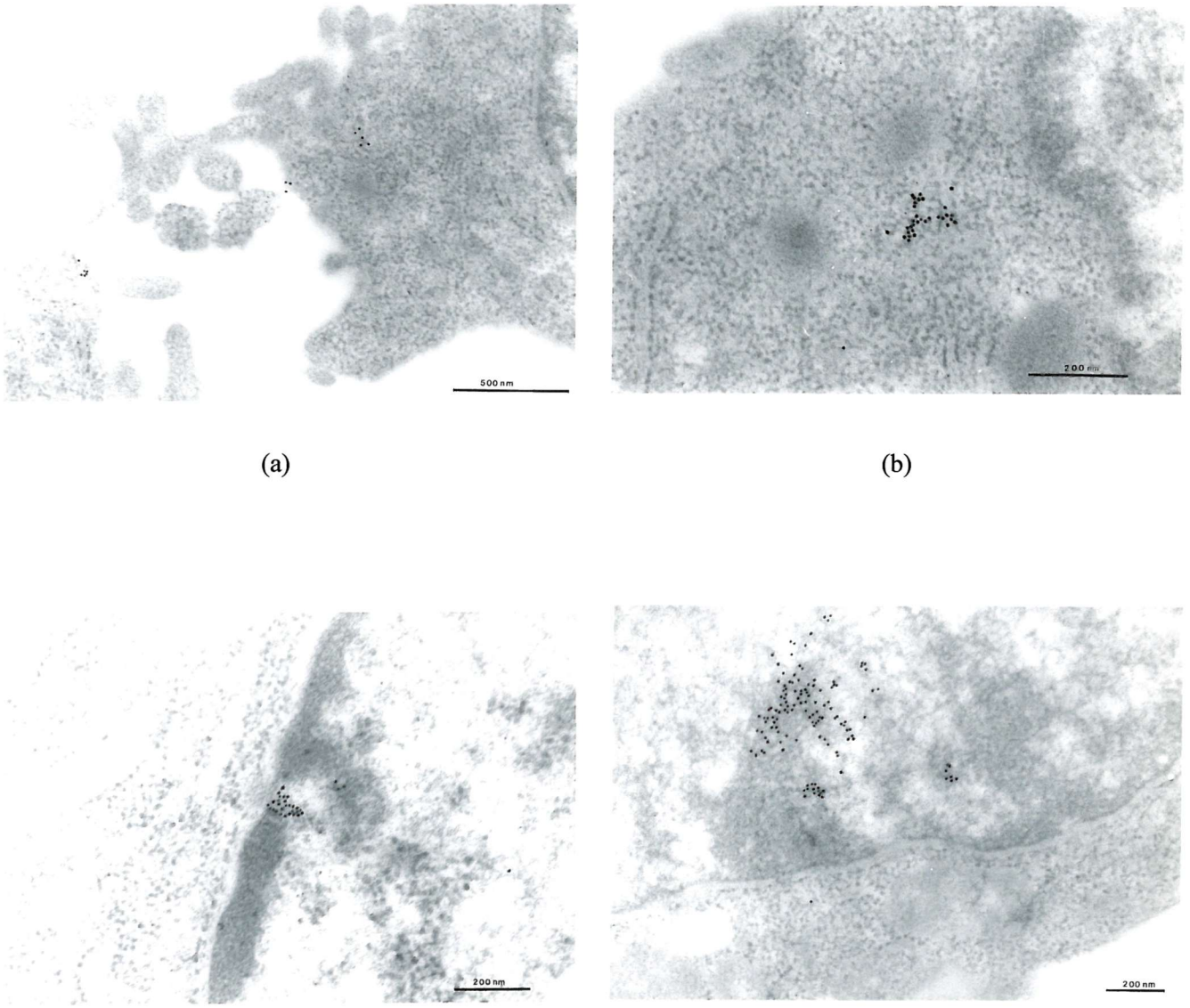
on the cell membrane, 36.6% within the cytoplasm, 7.9% at the nuclear membrane and 43.5% within the nucleus (Table 7). Typical immunogold distribution is shown (figure 14a-d). The nuclear distribution of immunogold is seen within the nuclear chromatin (14d). Control studies with preabsorbed α-CCKBR-Ser, serine spacer element peptide, and without primary antibody were all negative.

Table 7.

Distribution of Immunogold Particles within AR42J Cells after 1h Incubation with Immunogold Labeled CCK-B Receptor Antibody (anti-GRP1-Ser).

	Cell membrane	Cell matrix	Nuclear membrane	Nuclear matrix
No. gold particles	14.2 (+/- 0.97)	43.3 (+/- 2.32)	9.3 (+/- 0.81)	51.4 (+/- 3.32)
% distribution within a cell	12%	36.6%	7.9%	43.5%

(mean +/- SEM for 25 cells, each experiment repeated 5 times).



(a) (b)

(c) (d)

Fig. 14. Immunogold localisation of CCK-B receptor: (a) on plasma membrane of two cells; (b) cytoplasm; (c) nuclear pore; and (d) nuclear chromatin. Cells were incubated with α-CCKBR-Ser antibody for 1 h. Grids were incubated for 1 h with goat anti-biotin antibody conjugated to 10nm gold particles. Twenty-five cells/grid were counted by an independent observer.

5.4 Discussion

This study has identified the CCK-B receptor not only on the surface membrane, but also within the cytoplasm and nucleus of the AR42J pancreatic adenocarcinoma cell-line.

The internalisation of the CCK-B/gastrin receptor has recently been demonstrated in a number of gastrin-receptor expressing cell lines, including AR42J, using confocal laser scanning microscopy (134). The proposed mechanism of internalisation was via clarithin-coated pits. This mechanism has also been demonstrated for other G protein coupled receptors including gastrin-releasing peptide (135).

The nuclear expression of the CCK-B/gastrin receptor has not previously been described, however recent work would suggest that the possibility of nuclear translocation of the ligand gastrin or its receptor is not unique. There is corresponding evidence for other G-protein coupled receptors to suggest that epidermal growth factor (136), fibroblast growth factor (137) nerve growth factor (138), interleukin 1 (139), and insulin (140) can all directly or via their membrane receptor affect the cell cycle by translocation of the peptide and/or receptor to the nucleus.

The mechanism for nuclear translocation of the receptor has yet to be elucidated. It is known that G proteins are involved in endocytosis (141). Transport may involve actin or microtubular components of the cytoskeleton and Figure 14c would suggest translocation through the nuclear pore.

Within the nucleus we have seen areas of intense CCK-B receptor immunoreactivity located on chromatin, figure 14d, which may suggest specific binding

sites for regulation of the DNA. This has similarly been described for epidermal growth factor receptor (142).

In conclusion this study has demonstrated a specific CCK-B/gastrin receptor antibody (appendix 1) and identified the receptor not only on the cell membrane, but also within the cytoplasm and nucleus using immunoelectron microscopy and Western blotting. The nuclear expression of the receptor highlights the possibility of direct receptor-mediated regulation of cell function and cell proliferation.

Chapter 6:

SUMMARY AND FUTURE CONSIDERATIONS

Gastrin is a growth factor and has been shown to promote the growth of normal gastrointestinal mucosa as well as a variety of cancers including gastric, colonic, pancreatic, lung, hepatocellular and neuronal malignancies.

In vitro and *in vivo* studies have shown that neoplastic cells not only have the ability to respond to circulating forms of gastrin but also respond to the autocrine production of gastrin and its precursors. The CCK-B/gastrin receptor has been well described in many types of neoplastic cells.

Amidated gastrin binds to the CCK-B/gastrin receptor and activates multiple signal transduction pathways resulting in cellular proliferation. The precursor forms of gastrin bind to an as yet uncharacterised CCK-C receptor. This activates an even more poorly understood signal transduction pathway, which results in an even greater proliferative response than activation of the CCK-B receptor.

This thesis has demonstrated the proliferative effect of gastrin and precursor forms in hepatocellular cell lines as well as demonstrating the expression of CCK-B/gastrin receptor, gastrin and its precursor forms in hepatocellular cancers and pancreatic cancer suggesting an autocrine production of gastrin by these cancers.

Anti-gastrin agents have been shown to inhibit both the *in vivo* and *in vitro* proliferative effects of gastrin. The anti-gastrin immunogen G17DT raises antibodies to both amidated and glycine-extended forms of gastrin and abrogates the gastrin proliferative effect. CCK-B/gastrin receptor antagonists however, are only able to inhibit the proliferative effects of amidated gastrin and have no effect on precursor forms, confirming that these precursor forms bind to a separate receptor. The CCK-B/gastrin receptor can however be utilised for therapy. Rather than just blockading the receptor with specific antagonists, the receptor can be utilised for targeting with a view to cell death. Studies have demonstrated the efficacy of such an approach using

amidated peptide labelled with a radionuclide or antibody acting either as a therapeutic vector or by directly triggering apoptosis (143, 144).

In view of the poor response to available therapies for inoperable pancreatic cancer two recent phase II studies of the anti-gastrin agent G17DT have been completed. Both studies demonstrated good tolerability and anti-gastrin antibody induction due to G17DT. Those patients forming antibodies had significantly improved survival (145, 146). G17DT is now undergoing phase III studies in Europe and USA. Similar studies are also being undertaken using G17DT in gastric cancer and other studies are due to be initiated.

Gastrin therefore appears to be an important growth factor and targeting the peptide, its precursor forms, or its receptor represents novel and appropriate therapeutic targets.

Appendix 1:

SPECIFICITY STUDIES AND WESTERN BLOTTING FOR CCK-B/ GASTRIN RECEPTOR CONTRIBUTING TO DEMONSTRATION OF NEW SITES OF EXPRESSION OF THE CCK-B/GASTRIN RECEPTOR IN PANCREATIC ACINAR AR42J CELLS

Contents

A1.1 Introduction:

The following studies were performed in order to determine specificity of the ant-CCK-B/gastrin receptor antibody. Additionally Western blotting was performed in order to confirm the extra-nuclear and nuclear localisation of the antibody as seen on immunoEM (see chapter 5).

A1.2 Materials and Methods

1.2.1 Raising CCK-B/gastrin receptor (α-CCKBR-Ser Antibody)

(as per chapter 2)

α-CCKBR-Ser antibody is an affinity purified polyclonal antibody raised against the amino-terminal sequence of the CCK-B/gastrin receptor, as follows: peptide with the sequence KLNRSVQGTGPGPGASLSSPPPPC, comprising residues 5-21 of the CCK-B/gastrin receptor followed by a seven residue spacer at the C-terminus was custom synthesised (BACHEM, Torrance, CA, USA). The peptide was linked to diphtheria toxoid (DT) using the bifunctional cross-linking agent, 6-maleimido caproic acyl N-hydroxy succinamide ester (MCS, Sigma Chemical Co.) by a modification of methods previously described (23). Immunogens were formulated at 1.0mg/ml conjugate by emulsifying conjugate-bearing aqueous phase with Montanide ISA 703 adjuvant (Seppic, Paris, France) in a 30:70 (wt:wt) ratio of water:oil. New Zealand white rabbits were injected intramuscularly with 0.25ml/injection at 0, 4 and 9 weeks. Sera was prepared from blood collected subsequent to the third injection, and α-CCKBR-Ser antibodies were prepared by affinity purification of the sera over recombinant Protein A-Agarose (Boehringer Mannheim GmbH, Germany).

Three separate studies were performed to determine antibody specificity.

A1.2.2 ***Inhibition ELISA studies***

An inhibition ELISA was used to assess the specificity of the affinity purified antibodies for α-CCKBR-Ser peptide. The assays were run as follows: α-CCKBR-Ser -BSA conjugate was coated onto 96 well plates (Immulon U bottom) by overnight incubation of 50 µl of a 2 µg/ml solution of conjugate in glycine buffer (0.1M, pH=9.5) at 4°C. Affinity purified α-CCKBR-Ser antibody (at a final concentration of 10 ng/ml) was combined with various inhibitors (in 1:10 dilution series) and incubated for 1 hour at room temperature. The inhibitors included CCKBR epitope peptide, CCKBR epitope peptide + joined Ser spacer peptide, Ser spacer peptide, joined gastrin + spacer element peptide (hG17(9)-Ser), CCKBR + epitope + Ser spacer not joined, and buffer (no inhibitor). Incubation buffer consisted of PBS + 0.5% BSA + 0.05% Tween 20 + 0.02% NaN_3. Subsequent steps used the same buffer without BSA. The 96 well plates were washed free of non-bound α-CCKBR-Ser-BSA, and the Antibody + inhibitor mixtures were added (50 µl/well). After 1 hour, the plates were washed and a goat anti-rabbit Ig (H+L) alkaline phosphatase conjugate (Zymed) was added (1:2000 dilution). After 1 hour incubation, the plates were washed to remove non-bound reagent, and 50 µl/well of pNPP substrate (Sigma) solution (1 mg/ml) was added in substrate buffer (PBS + 0.1 mg/ml $MgCl_2$ + 10% diethanolamine + 0.02% NaN_3). Following a 60 minute incubation, absorbance was measured on a MRX reader (Dynatech Laboratories). Samples were run in duplicate, and means were calculated for each concentration. Background binding (established with affinity purified rabbit anti-GnRH antibodies) was subtracted from all values, and the % Inhibition relative to no inhibitor added (anti-GRP1 Ab + buffer) was

calculated for each inhibitor tested: % Inhibition = (100)(1-(($A_{uninhibited}$-$A_{inhibited}$)/$A_{uninhibited}$), where A = Absorbance.

A1.2.3 ***Radioligand binding inhibition studies***

AR42J cells were seeded into plastic LP3 tubes at a cell concentration of $5x10^5$ in a 50µl volume of 50mM HEPES buffer, pH 7.0 (containing 5mM $MgCl_2$, 115mM NaCl, 0.05% Soya trypsin inhibitor, 0.1% bacitracin, 1mM EGTA, 1mM PMSF, 0.2% BSA, 5µM pepstatin, 2mM 1,10-phenanthroline and 10% glycerol [all reagents, Sigma]). PMSF was added fresh each time the buffer was used. ^{125}I G17 (NEN Dupont, specific activity 2200Ci/mmol) was added to each tube at a concentration of $1x10^{-10}$ M. The radiolabelled G17 was competitively displaced using (i) unlabelled G17I (Sigma) at concentrations from $5x10^{-10}$ to $5x10^{-6}$M, (ii) the affinity purified rabbit α-CCKBR-Ser antibody or (iii) normal rabbit antiserum (Dakopatts). The antisera were prepared to a protein concentration of 0.5mg/ml and dilution's of 1:2, 1:4, 1:5, 1:6, 1:10, 1:50 and 1:100 were prepared in 50mM HEPES buffer. The tubes were incubated for 90 mins at room temperature. After which 3mls ice-cold phosphate buffered saline was added to each tube and centrifuged at 463g for 10 mins. This washing procedure was repeated and tubes were counted on a Gamma counter (counting efficiency 87%). Total CPM per tube and background radioactivity were measured and used in the calculation to measure specific radioactivity displaced from the AR42J cells.

A1.2.4 ***Immunofluorescence detection of binding***

Binding of the α-CCKBR-Ser specific antibodies to AR42J cells was assessed by immunofluorescence. AR42J cells were harvested from T-75 flasks and washed twice with buffer (PBS with 0.02% NaN_3) and centrifuged (400 X g for 7 min). The

cells were kept at 0-4°C for all manipulations. A single cell suspension was prepared in buffer, and the cell concentration was adjusted to 10^6 cells/ml. The cell suspension was added to 1.5 ml microfuge tubes (1 ml/tube). The cells were pelleted by centrifugation and supernatants were aspirated. The cells were resuspended in buffer (0.1 ml/tube) containing peptide inhibitors (1.0 mg/ml). The inhibitors included CCKBR-Ser, GnRH, hG17(9)-Ser and buffer (no inhibitor). Antibodies, including the rabbit α-CCKBR-Ser (100 μg/ml), affinity purified rabbit anti-DT (negative control, 100 μg/ml), mouse anti-AR42J antiserum (positive control, 1:100 dilution, heat inactivated) or normal mouse serum were added to the appropriate tubes and the contents were mixed. The cells were incubated for 1 hour, with occasional mixing. The cells were then washed three times with buffer, and 0.1 ml of fluorescein labeled goat anti-rabbit IgG (Antibodies Incorporated) (diluted 1:50) was added per tube. The cells treated with mouse sera were developed with a fluorescein-anti-mouse IgG reagent (Zymed). The cells were re-suspended by vortexing, then incubated for 1 hour. The cells were again washed three times, then re-suspended in glycerol:PBS (1:1, v:v), 50 μl/tube. Wet mounts were prepared with the contents of each tube, and the cells examined using a Laborlux 12 fluorescent microscope (Leitz). Fluorescence was scored on a scale of 0 to 4, with 0 representing background fluorescence (obtained with the normal mouse serum) and 4 representing maximal fluorescence (obtained with the mouse anti-AR42J positive control antiserum).

A1.2.5 **Western Blotting**

AR42J Cells (from 30 x T75 flasks) were resuspended in 5ml of homogenisation buffer (1mM sodium hydrogen carbonate, 2mM magnesium chloride, 1nM phenyl methyl sulphonyl fluoride, 40mM sodium chloride, 10ul leupeptin, 1uM pepstatin, 5nM

EDTA [Sigma]). Homogenisation was carried out by 5 bursts of 5sec duration. For extranuclear membranes, tissue debris was pelleted by centrifugation at 500g, 7min, 4°C. The pellet was discarded and the supernatant centrifuged at 500g, 7min, 4^0C to remove further debris. The supernatant was then re-centrifuged at 48000g, 60min, 4^0C. The extra-nuclear membrane preparation (pellet) was suspended in Tris/NP40 solution (0.1M Trizma, 0.5% Nonidet P40 [Sigma]).

For nuclear membrane preparations, following homogenisation in a second homogenisation buffer (25mM Tris-HCl, pH 7.4, 0.1% Triton 100, 0.32M sucrose, 3mM MgCl2, 2mM EGTA, 0.1mM spermine tetrahydrochloride, 2mM PMSF, 10mM bezomidine hydrochloride, 3mM aminoacetonitrile hydrochloride [Sigma]), tissue debris was pelleted by spinning at 700g, 10min, 4^0C. The pellet was resuspended in 55% sucrose (0.2M in HPLC water). This was then spun at 60000g, 60min, 4^0C. The pellet was washed with 0.4% Nonidet P40 in homogenisation buffer (minus triton 100). The pellet was spun at 700g, 15min, 4^0C and resuspended in homogenisation buffer (minus triton 100).

Protein content was determined by the Lowry method (using a kit from Pierce & Warriner, Rockford, IL,USA). Samples containing 10-15ug protein were loaded onto a 8-16% Tris/glycine gradient PAGE gel (Novex R and D systems) in Tris glycine buffer and run for 90min (125V constat). The gel was equilibrated for 10min in Transfer buffer (Novex) and then blotted onto PUDF membrane. The membranes were blocked with 0.5% casein (Sigma) for 60min and incubated with α-CCKBR-Ser antibody (with and without preabsorption) for 60min. Antibody binding was detected by the avidin:biotin-peroxidase complex method using diamino-benzidene as the substrate.

A1.3 **Results**

The results of the inhibition ELISA studies are shown in Figure Appx. 1, which presents the percent inhibition of antibody binding as a function of inhibitor concentration. As can be seen in the Figure, the CCKBR-Ser peptide fully inhibited antibody binding to α-CCKBR-Ser-BSA. Roughly 60% inhibition was attained with the CCKBR epitope peptide, which does not contain the Ser spacer sequence, and by an equimolar mixture of CCKBR epitope peptide + Ser spacer. The failure of these peptides to produce full inhibition suggests that a proportion of the antibodies were specific for an epitope(s) comprising elements of both the CCKBR and the Ser spacer sequences. No inhibition was obtained by either the Ser spacer sequence itself or by an unrelated peptide bearing the joined gastrin-spacer peptide, consisting of the amino-terminal nine residues of hG17 followed by the Ser spacer. These ELISA results demonstrate that the affinity purified antibody preparation was specific for the CCKBR-Ser peptide, and that 60% of the binding activity was directed against the gastrin receptor epitope component of the peptide.

The results of the radioligand binding inhibition tests showed that unlabeled G17 displaced ^{125}IG17 from AR42J cells with an IC_{50} between $5x10^{-9}$ and $5x10^{-10}$M. Rabbit α-CCKBR-Ser antiserum, but not normal rabbit antiserum, displaced ^{125}IG17 at dilution's up to 1:10 (relating to a protein concentration of 50μg/ml) with the level of displacement at a 1:2 dilution relating to a cold G17 concentration of $1.34x10^{-9}$M. These results confirmed the cell surface expression of gastrin receptor by the AR42J cells and the competitive displacement of ^{125}IG17 by α-CCKBR-Ser antibody. Figure 2 demonstrates the inhibition binding curve of ^{125}IG17 by G17 on AR42J cells.

The results of the immunofluorescence tests are presented in Table Appx. 1. As can be seen, AR42J cells treated with α-CCKBR-Ser antibodies in the absence of

peptide inhibitors fluoresced strongly, indicating that the antibody bound to the cells (figure Appx. 3). Rabbit anti-DT antibodies did not produce fluorescent staining, demonstrating that the staining observed with the α-CCKBR-Ser antibodies was not a consequence of non-specific cell surface binding by rabbit immunoglobulin. Moreover, the binding was shown to be specific for the CCKBR-Ser peptide. Addition of CCKBR-Ser fully inhibited binding, whereas unrelated peptides, including hG17(9)-Ser and GnRH, failed to inhibit. As the CCKBR epitope comprises residues 5-21 of the CCK-B/gastrin receptor, it was concluded that the α-CCKBR-Ser antibodies were specific for the gastrin receptor expressed by AR42J cells.

Western blotting for CCK-B/gastrin receptor (figure Appx. 4) demonstrated that the extra-nuclear membrane extracts showed a predominantly single immunoreactive band localising at about 60 kDa (lane B). The nuclear extracts showed two doublet immunoreactive bands localising at 58 and 66 kDa (lane C). Control studies using preabsorbed antibody showed no staining.

Figure Appx. 1

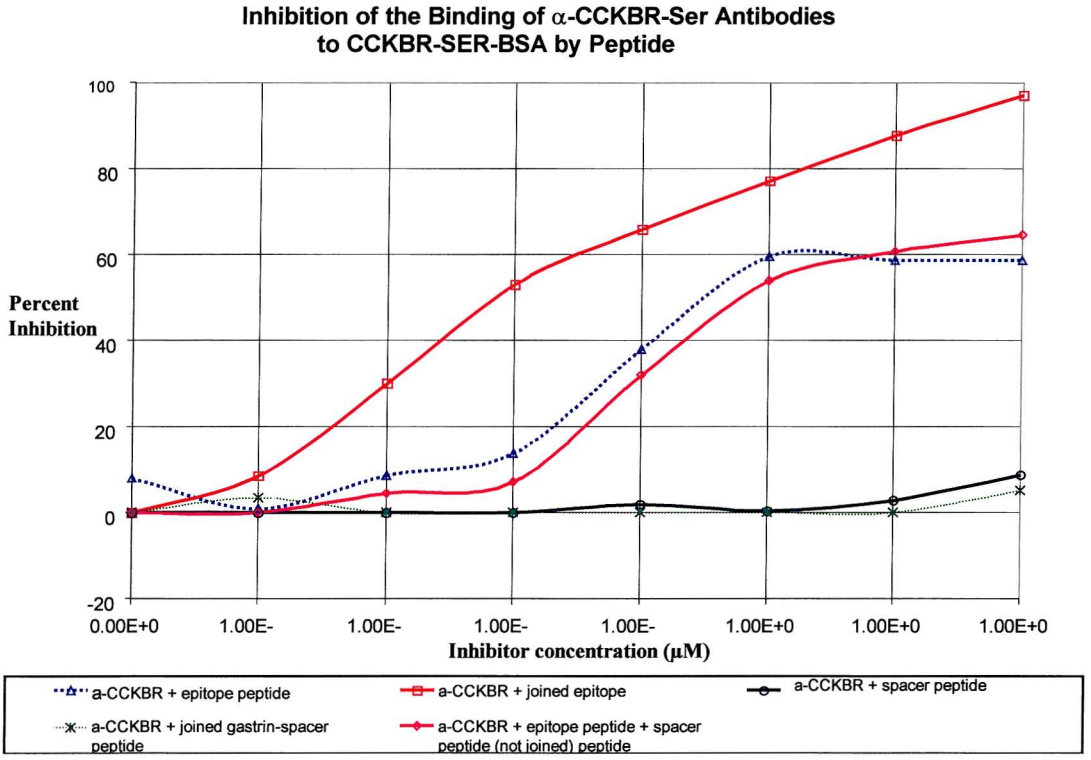


Figure Appx 1

Inhibition of the binding of α-CCKBR-Ser antibodies to CCKBR-Ser by peptide inhibitors. GRP-Ser-BSA conjugate was coated onto 96 well plates. Inhibition ELISAs were performed by combining α-CCKBR-Ser antibody with inhibitors including CCKBR epitope peptide + joined Ser spacer, CCKBR epitope peptide, Ser spacer peptide, joined gastrin [G17(9)]-Ser spacer peptide, CCKBR peptide + Ser spacer not joined, and buffer (no inhibitor).

Figure Appx. 2
INHIBITION BINDING STUDY OF ^{125}I-G17 VERSUS G17 ON AR42J CELLS

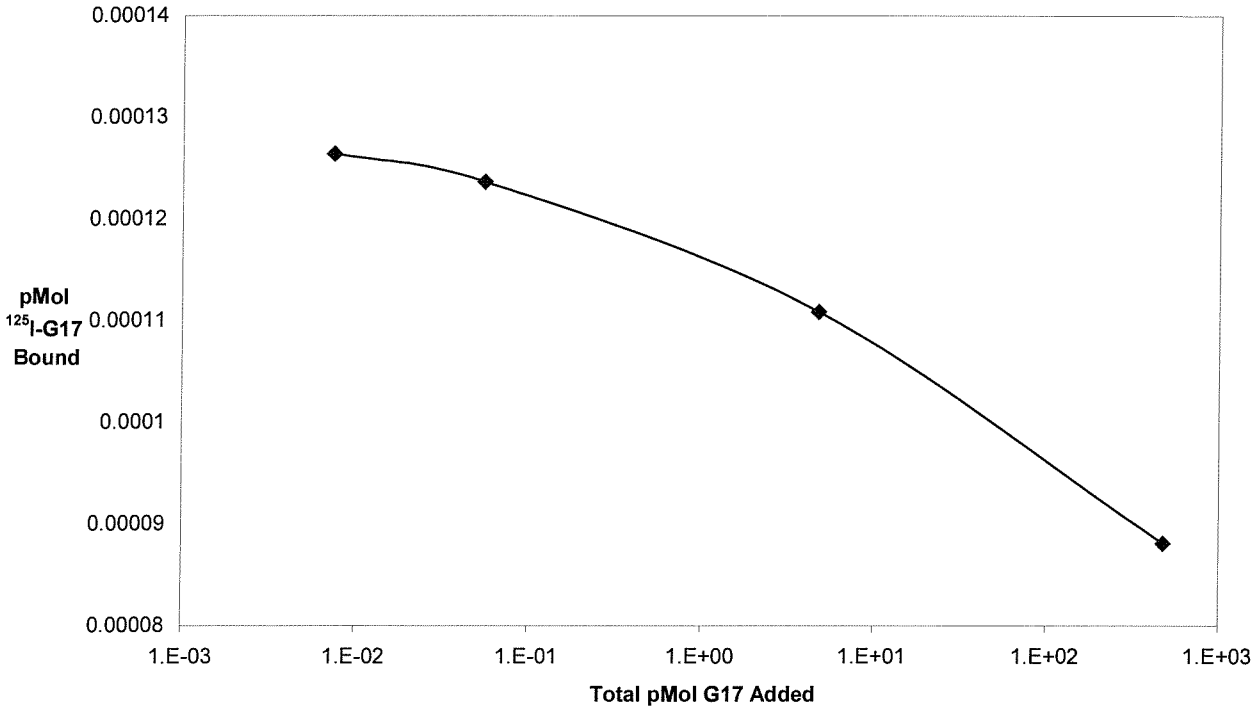


Figure Appx. 2

Inhibition binding study of ^{125}I-G17 versus G17 on AR42J cells. ^{125}I-G17 was added to each tube at a concentration of 10nM. The radiolabelled G17 was competitively displaced using unlabelled G17 at $5x10^{-10}$ to $5x10^{-6}$M.

Table Appx. 1

Specificity of Binding of Antibodies to AR42J Cells as Assessed by Immunofluorescence

Antibody	Inhibitor			
Preparation	CCKBR-Ser	hG17(9)-Ser	GnRH	Buffer
Rabbit anti-CCKBR-Ser	0	3+	2+	3+
Rbt anti-DT	0.5+	0.5+	0.5+	0.5+
Mouse anti-AR42J				4+
Normal Mouse Serum				0

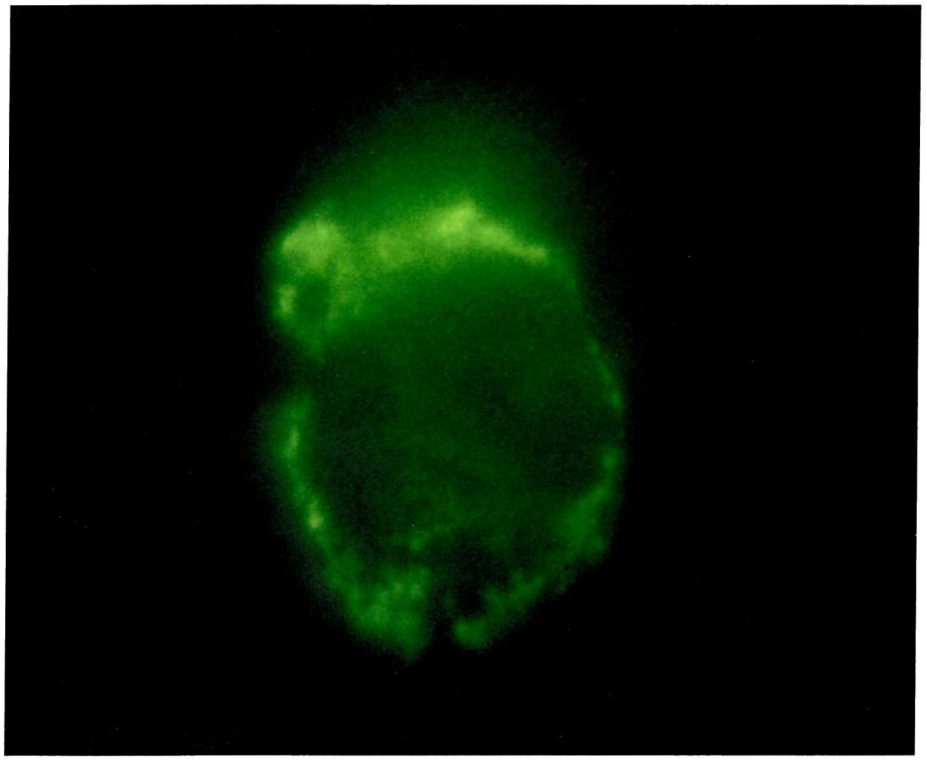


Figure Appx. 3

Immunofluorescence microscopy of α-CCKBR-Ser binding to the membrane of AR42J cells. α-CCKBR-Ser (100ug/ml) was incubated with AR42J cells for 1 hour. Cells were developed with a fluoroscein-anti mouse IgG and subsequently examined using a Laborlux 12 fluorescent microscope.

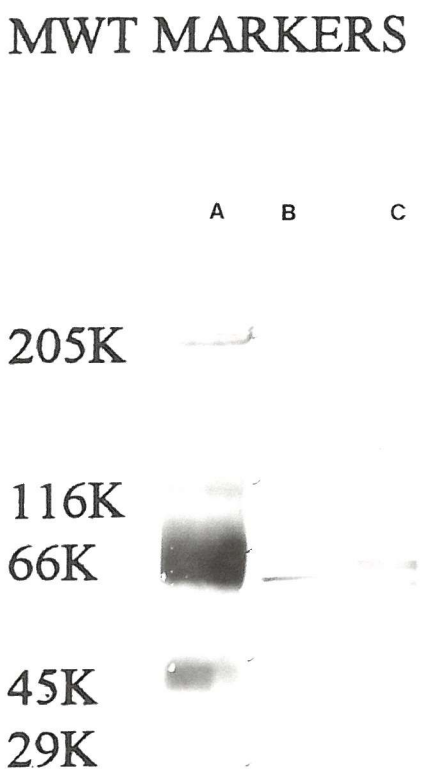


Figure Appx. 4. Western blotting for CCK-B/gastrin receptor. Extra-nuclear and nuclear fractions of AR42J cells were prepared. The molecular weight markers localised at 205, 116, 66, 45 and 29 kDa (lane A). The extra-nuclear membrane extract showed a predominantly single immunoreactive band localising at about 60 kDa (lane B). The nuclear extracts showed two immunoreactive bands localising at 58 and 66 kDa (lane C).

A1.4 **Discussion**

A series of tests were conducted to assess the specificity of the rabbit polyclonal antibody, α-CCKBR-Ser. The antibodies were first shown to be specific for the CCKBR-Ser peptide by ELISA, wherein the binding of the antibodies to a CCKBR-Ser-BSA conjugate was fully inhibited by free CCKBR-Ser peptide. Partial inhibition was obtained with the CCKBR epitope and no inhibition was obtained by either the Ser spacer peptide or by an unrelated peptide bearing the Ser spacer. The capacity of the antibodies to specifically bind gastrin receptor was then tested against AR42J cells. Cellular expression of the gastrin receptor was first established by radioligand inhibition tests, wherein the binding of ^{125}I-hG17 to AR42J cells was competitively inhibited by non-labeled hG17 and by α-CCKBR-Ser antibody. It was then shown by immunofluorescence that the α-CCKBR-Ser antibodies bound to the AR42J cells. The cell surface binding was inhibited by CCKBR-Ser, but not by either hG17(9)-Ser or GnRH. Thus, it was demonstrated that the affinity purified α-CCKBR-Ser antibodies were specific for CCKBR peptide, and that the binding of these antibodies to CCK-B/gastrin receptor-bearing AR42J cells was specifically inhibited by CCKBR-Ser peptide.

The Western blots detected the expression of receptor protein in both extra-nuclear and nuclear extracts with molecular weights ranging from 58-66 kDa.

Although the CCK-B receptor originally described on canine parietal cells had a molecular weight of 74 kDa (147), there is a range of molecular weight proteins described for the CCK-B receptor. A truncated form, as a result of a novel exon 1b, is known to exist (148) however it is unlikely to account for the lower molecular weight receptor we describe as this antiserum is unable to detect the truncated CCK-B receptor. There is however evidence to suggest that internalised CCK-B receptors have a lower

molecular weight and it is possible that there may be partial degradation of such internalised receptors (149). Another possibility is that different receptor molecular weights may be related to glycosylation of the receptor. CCK-B receptors in the calf pancreas are different in milk fed animals compared to ruminant calves as a result of deglycosylation of receptor components (150). In the guinea pig, colonic membranes expressed a wide range of gastrin binding proteins of 33, 45, 80 and 250kDa (151). A low molecular weight, 45kDa, receptor isoform on Swiss 3T3 cells has been shown to bind both glycine extended and carboxy-amidated gastrin in a non-discriminative manner (152). In the present study the lower molecular weight form was not shown on extra-nuclear membrane preparations which may argue against it being a distinct receptor.

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PUBLICATIONS RELATED TO THIS THESIS

Chapter 2:

Caplin M, Khan K, Grimes S, Michaeli D, Savage K, Pounder R, Dhillon A. Effect of gastrin and anti-gastrin antibodies on proliferation of hepatocyte cell lines. *Dig Dis Sci* 2001; 46(7): 1356-66

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Chapter 5:

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