Pergamon

European Journal of Cancer, Vol. 35, No. 1, pp. 125–132, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(98)00276-7

Original Paper

High Gastrin Releasing Peptide Receptor mRNA Level is Related to Tumour Dedifferentiation and Lymphatic Vessel Invasion in Human Colon Cancer

J.-C. Saurin,[1] J.-P. Rouault,[2] J. Abello,[1] F. Berger,[3] L. Remy[1] and J.A. Chayvialle[1]

[1]Inserm U45, Hôpital Edouard-Herriot, Pavillon H, 69437 Lyon, Cedex 3; [2]Inserm U 453, Centre L. Bérard; and [3]Service d'anatomie pathologique, Hôpital E. Herriot, Lyon, France

The neuropeptide bombesin stimulates tumour cell proliferation *in vitro*. Through pharmacological testing, 20–40% of human colorectal tumours have been shown to be equipped with bombesin/gastrin releasing peptide receptor (GRP-R). The aim of the present study was to test whether GRP-R expression is correlated with tumour characteristics and usual prognostic factors in colorectal adenocarcinomas. A sensitive reverse transcription (RT)-competitive polymerase chain reaction (PCR) method was validated by studying GRP-R mRNA in separated layers of normal colonic wall, and GRP-R mRNA levels (in parallel with binding studies) in colon cancer cell lines LoVo and Caco-2. GRP-R mRNA levels were then determined in 29 surgical tumour specimens and the results compared with tumour histology and, using histochemistry, with the accumulation of p53 protein and a Ki-67 cell proliferation index. The mRNA was not detected in normal colonic epithelium, whereas a distinct signal was observed after amplification in 27/29 (93%) tumour specimens. Estimates of mRNA levels in the 27 positive tumours ranged from 52 to 8000 amol/0.25 μg total RNA, and were significantly higher in poorly/moderately differentiated tumours ($P < 0.05$) and in tumours with lymphatic vessel invasion ($P < 0.01$). There was no relationship with p53 accumulation or to the proliferation index. Our results show that GRP-R mRNA can be detected in most colorectal tumour specimens, and suggest a link between high mRNA levels and both tumour dedifferentiation and lymph vessel invasion, but not proliferation. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: bombesin, gastrin releasing peptide receptor, colorectal adenocarcinomas, colonic epithelium, competitive PCR, lymphatic vessel invasion, differentiation, proliferation, p53
Eur J Cancer, Vol. 35, No. 1, pp. 125–132, 1999

INTRODUCTION

THE TETRADECAPEPTIDE bombesin and its mammalian counterpart gastrin releasing peptide (GRP) have been shown to stimulate proliferation of cancer cell lines both *in vitro* and *in vivo* [1–3]. Interestingly, a growth promoting autocrine loop involving GRP and its receptor has been demonstrated in small cell lung cancer cell lines and suspected in pancreatic cancer cell lines [1, 4]. Besides growth promoting effects, bombesin also stimulates invasion of PC3 prostate cancer cells in the Boyden chambers assay [5] and increases metastasis of azoxymethane-induced rat colon cancer to the peritoneum *in vivo* [6].

Correspondence to J.-C. Saurin, e-mail: saurin@lyon151.inserm.fr
Received 1 Apr. 1998; accepted 1 Jun. 1998.

At the plasma membrane level, peptides of the bombesin family interact with three G protein-coupled receptor subtypes in mammalian cells. The neuromedin B (NMB)-preferring subtype has been detected in brain and in the oesophageal *muscularis mucosae* [7]. The GRP-preferring subtype, present in brain, intestine and pancreas, recognises equally bombesin and GRP, but has a lower affinity to NMB [8]. For the bombesin receptor subtype 3, present in testicular and lung cancer cell lines, the first synthetic specific ligand has recently been discovered [9]. In cancer cell lines, bombesin receptors are predominantly of the GRP-preferring subtype [1, 10].

Previous binding studies have shown the presence of bombesin/GRP receptors (GRP-R) in approximately 30% of both human colorectal adenocarcinoma specimens and

colorectal cancer cell lines, but not in normal colonic mucosa [10, 11]. Contrasting with tumour cell lines, the role of the GRP-R in human colorectal tumours is unknown, and its expression has not been tested against the many oncological parameters that are presently used as prognostic factors. In this setting, we developed a sensitive reverse transcription-competitive polymerase chain reaction amplification (RT-competitive PCR) method to compare GRP-R mRNA levels in a series of tumour specimens. In order to validate the technique, GRP-R mRNA levels were studied in parallel with bombesin binding in colon cancer cell lines LoVo and Caco-2, and the presence or absence of the mRNA was tested in separated layers of normal colonic wall.

MATERIALS AND METHODS

Cell lines, tracer preparation and binding assays

The human colon cancer cell line Caco-2 (ATCC HTB 37) and two clones of the human colon cancer cell line LoVo were used. Clone E2 generates well differentiated tumours when xenografted to immunosuppressed newborn rats, whereas clone C5 generates less differentiated islets of tumours lined with discontinuous basement membrane [12]. Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (100 IU/ml) and streptomycin (50 μM), and were sampled at 90% confluence.

Five micrograms ^{4}Tyr-bombesin were dissolved in 10 μl 0.5 M ammonium acetate, pH 5.5, with 1 mCi [^{125}I]-Na (Amersham, Les Ulis, France), 5 μg lactoperoxidase (Sigma, Saint-Quentin-Fallavier, France), and 5 μl 0.06% hydrogen peroxide for 15 min. The radiolabelled peptide was purified by reverse-phase high-performance liquid chromatography (HPLC, C18 μBondapak) in isocratic conditions (27% acetonitrile in water and 0.1% trifluoroacetic acid). The specific activity of the radioactive ligand was approximately 2000 Ci/mmol.

Cells were harvested from culture flasks with trypsin/ethylene diamine tetra acetic acid (EDTA), washed twice in 5-fold volume media, spun down at 1000 ***g*** for 5 min and resuspended in binding buffer (Hepes 50 mM, sucrose 0.25 M, NH_4Cl 10 mM, phenylmethylsulphonyl fluoride 0.5 mM, $MgCl_2$ 2 mM, 1% bovine serum albumin (BSA), bacitracin 0.1%, phenantroline 1 mM, soybean trypsin inhibitor 0.2%, pH 7.5). For each experiment, 2×10^6 cells were used in a final volume of 120 μl. [^{125}I]-^{4}Tyr bombesin was added at a concentration of 75 pM (1.5×10^5 cpm/sample). Incubation was performed for 30 min at 25°C under constant agitation (equilibrium binding conditions). The reaction was stopped by the addition of phosphate buffered saline (PBS)–0.2% BSA at 4°C and the cells were centrifuged at 1600 rpm for 5 min. The cells were washed once with PBS–BSA, spun down, and the pellet was counted in a gamma-counter. Specific binding was calculated as the difference between the amount of [^{125}I]-^{4}Tyr bombesin bound in the absence (total binding) and the presence (non-specific binding) of 1 μM unlabelled bombesin.

Tissue sampling for total RNA preparations and RNA analysis

Ten samples of total colonic wall were obtained after left hemicolectomy from patients with diverticulitis ($n = 4$) or left colon cancer ($n = 6$). The colonic wall was taken at a minimal 5 cm distance from the diseased area. Ten millilitres of isotonic saline were injected in the submucosa ('strip-biopsy' technique) [13] in order to separate the epithelium and submucosa from the muscle layer. The superficial layer was severed, washed with ice-cold isotonic saline and its epithelial side immersed in Trizol reagent (Life Technologies, Cergy-Pontoise, France) for 15 min for cell disruption. RNA was extracted from this solution (epithelial fraction). The remaining fragment (submucosal fraction) and a fragment of muscle layer (muscle fraction) were then carefully scraped and was half used for RNA extraction and half for histological control.

In 29 other patients (15 men and 14 women, median age 65 years), specimens of colorectal adenocarcinomas were sampled in the Pathology Department immediately after removal. Tissue aliquots (50–200 mg) were dissected and immediately frozen in liquid nitrogen. Samples were taken in tumour areas showing no necrotic or scirrhous pattern.

For total RNA preparation, $5–10\times10^6$ adherent cells (cell lines), fractions of normal colonic wall and tumour specimens were lysed in Trizol reagent and RNA extracted following the manufacturer's instructions. To ascertain that equivalent amounts of intact RNA were analysed, total RNA from each sample was subjected to electrophoresis, blotted to nitrocellulose–Nylon filters and probed with a 2 kb human β-actin cDNA (Clontech, Ozyme, Montigny-le-Bretonneux, France). The latter step was omitted in the case of normal colon specimens, as the β-actin mRNA has been shown to be overexpressed in muscle compared with epithelial extracts [14].

Analysis of GRP-R mRNA expression

The relative GRP-R mRNA levels in cell lines and colorectal tumours were measured using a RT reaction followed by competitive PCR (RT-competitive PCR). The method is based on the coamplification of known amounts of a competitor DNA with the target cDNA, after cDNA synthesis from equivalent total RNA amounts [15].

Construction of the competitor. The GRP-R cDNA sequence (nucleotides − 115 to 469), amplified with primers B and C (Figure 1), was subcloned in Bluescript vector (Stratagene, Ozyme, France). The competitor was generated by deleting a 114 bp long Xbal/Smal fragment (nucleotides − 4 to 109) in the GRP-R sequence (Figure 1). This new construct containing the deleted GRP-R sequence was used as the competitor in the PCR reactions. Working solutions of the competitor were prepared by serial dilutions in H_20.

RT–PCR and RT-competitive PCR. First strand DNA synthesis was performed from 1 μg total RNA. In a final volume of 20 μl, RNA was denatured for 2 min at 72°C with 100 pmol of primer A (Figure 1), then incubated for 1 h at 42°C with 200 U of reverse transcriptase (MMLV RT Superscript, Life Technologies), 40 U of RNAse inhibitor (Boehringer Mannheim, Meylan, France), 9.5 mM dithiothreitol (DTT), 0.5 mM dNTP, in RT buffer (Life Technologies). After the reaction, 1 μl of the RT medium was added to a PCR mix [amplification buffer (Appligene, Illkirch, France), 50 pmol of sense and antisense primers B and C, 0.2 mM dNTP, and 1 U Taq polymerase (Appligene)] in a final volume of 97 μl. Three microlitres of known amounts of the competitor were then added to the PCR mix. For each sample, six to 10 PCRs with decreasing amounts of competitor (from 4×10^6 to 0.25 amol/tube) were performed in separate tubes, each subjected to 30 cycles of amplification (Perkin Elmer Cetus thermal cycler), including denaturation

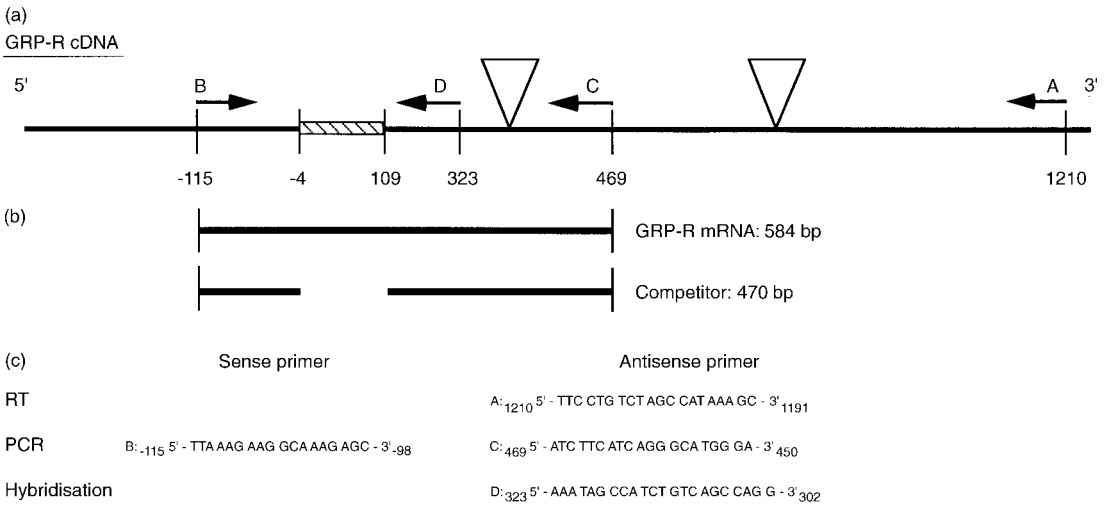

Figure 1. Gastrin releasing peptide receptor (GRP-R) cDNA, oligonucleotides and products of reverse transcription (RT)-competitive polymerase chain reaction (PCR). (a) Human GRP-R cDNA [8] with the position of the four oligonucleotides used for RT (A), PCR amplification (B, C) and specific hybridisation of PCR products (D), respectively. Arrows indicate the 5′–3′ orientation. The target sequence of the PCR (nucleotides − 115 to 469) overlaps the first intron of the gene (first triangle). The hatched bar represents the 114 bp deletion performed to obtain the competitor. The coding sequence of the cDNA corresponds to nucleotides 0 to 1154 [8]. (b) Size of the two PCR products. (c) Sequence of the four oligonucleotides.

(94°C, 1 min), hybridisation (59°C, 1 min) and elongation (72°C, 1 min). The absence of contaminants was checked by performing 60 cycle reactions (i) without added cDNA template and (ii) with samples in which reverse transcriptase was omitted. The localisation of the PCR primers (Figure 1b and c), flanking the first exon of the GRP-R gene, precluded amplification of genomic DNA.

For normal colonic wall extracts, RT–PCR was performed in the same conditions, but without the competitor. In these cases, RT of the GRP-R and β-actin mRNAs was performed simultaneously, with separate PCR amplification of the β-actin cDNA (human β-actin control amplimer set, Clontech).

Analysis of PCR products. The PCR products were separated on 1.5% agarose gels, blotted to nitrocellulose–Nylon filters, heat-crosslinked, and hybridised with 10^6 cpm/ml [^{32}P]-labelled oligonucleotide D (Figure 1) overnight at room temperature in 40% formamide, 5×SSC, 20 mM Tris pH 7.4, 10% dextran, 1×Denhardt's. Blots were then washed in 2×SSC, 0.1% sodium dodecylsulphate (SDS) twice at room temperature for 10 min and twice at 37°C for 10 min. The membranes were exposed to Kodak X-OMAT AR films for 2–72 h at − 80°C. The intensities of the hybridisation signals obtained with the two PCR products (GRP-R mRNA and competitor) were visually compared. The amount of GRP-R mRNA in the tested sample was estimated from the amount of competitor yielding an equivalent hybridisation signal after amplification (competition equivalence point) [15] or by the mean of the two values when the equivalence was localised between two consecutive amounts of the competitor. The sensitivity limit of the competitive PCR method, evaluated using the competitive template, was 25 amol (not shown).

Histology and immunohistochemistry studies

The tumour and adjacent wall were fixed in Bouin, embedded in paraffin, and processed for routine histology. Histological sections from the whole tumour were reviewed blindly for three parameters: (i) depth of invasion and node involvement, used for TNM staging along with the presence or absence of distant metastases [16]; (ii) differentiation, according to the predominant growth pattern (20 well differentiated, grade I, and nine moderately or poorly differentiated, grade II or III, adenocarcinomas) [17]; (iii) lymphatic vessel invasion, defined as tumour infiltration within an endothelial-lined space devoid of mural smooth muscle and elastic fibres, and located at least one high-power field from the border of the infiltrating tumour mass [18].

A section comprising the tumour and adjacent colonic wall was selected for immunohistochemistry. Mouse monoclonal antichromogranin A antibody (Boehringer, 1/200), mouse monoclonal anti-p53 antibody (Dako-p53, DO7, 1/25), and mouse monoclonal anti-Ki-67 antibody (Mib-1, Immunotech, 1/100) were used. For Mib-1 staining, the sections were incubated with 0.05% trypsin in citrate buffer (0.01 M, pH 6.0) twice for 5 min, followed by microwave treatment (three times 5 min) at 600 W in citrate buffer. For p53 staining, only microwave treatment (three times 5 min) was used. Binding of primary antibodies was detected using the immunoperoxidase method (streptavidin-biotin, Dako duet). Nuclei were counterstained with haematoxylin. Staining of adjacent normal colonic crypts showed chromogranin A positive cells (lower third), Mib-1 immunoreactive cells (basal part), but no p53 immunoreactive cells (not shown). No staining was observed in sections treated identically but without addition of the primary antibodies. Stained tumour sections were used to calculate a proliferation index and a p53 index (number of positive cells per 100 tumour cells) by counting at least 1000 cells under high magnification (40×).

Presentation of the results

GRP-R mRNA levels are the mean of four to eight determinations in cell lines and of two to five separate determinations in tumour extracts. To compare GRP-R mRNA levels with tumour characteristics, exact permutation tests were used for uni- and multivariate analyses (Statxact version 3 for Windows 1995, Cytel Software Corporation). A P value < 0.05 was considered statistically significant.

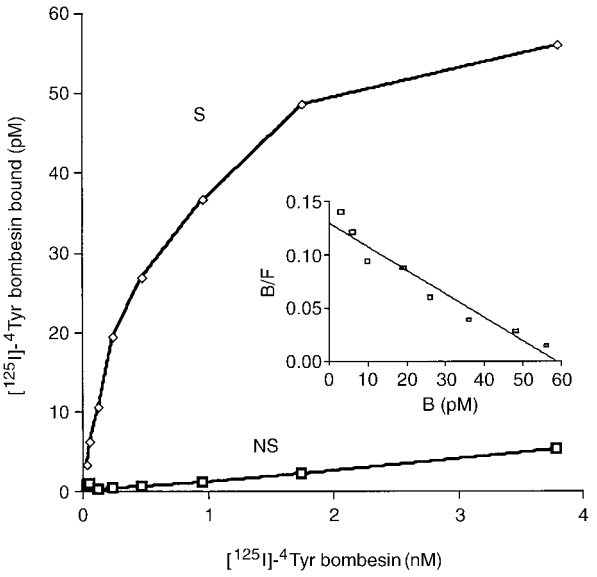

Figure 2. Concentration dependence of [^{125}I]-^{4}Tyr bombesin binding and corresponding Scatchard plot. LoVo E2 cells (2×10^{6}/tube) were incubated at 25°C for 30 min with the indicated concentrations of [^{125}I]-^{4}Tyr bombesin. Values are means of triplicate experiments. S, specific binding; NS, non-specific binding.

RESULTS

GRP-R binding and mRNA levels in colon carcinoma cell lines

Expression of GRP-R was first determined through binding experiments in LoVo E2, LoVo C5 and Caco-2 cells. Specific binding of [^{125}I]^{4}Tyr bombesin to LoVo E2 and LoVo C5 cells, but not to Caco-2 cells, was observed. The concentration dependence and saturability of [^{125}I]-^{4}Tyr bombesin binding to LoVo E2 cells (Figure 2) showed a single saturable site, indicating the presence of a homogeneous population of receptors with a high affinity constant ($K_d = 0.45$ nM, $B_{max} = 60$ pM). Maximal specific [^{125}I]-^{4}Tyr bombesin binding was 4.6 times lower by C5 than by E2 cells (respective density E2, 2580 receptors/cell; C5, 560 receptors/cell).

In LoVo E2 cells, RT–PCR amplification of the GRP-R mRNA yielded the expected 584 bp product, as visualised on ethidium bromide stained gels and after hybridisation of Southern blots with a specific oligonucleotide. Sequencing of the cloned PCR product confirmed that the amplified sequence was strictly identical to GRP-R cDNA (data not shown). In an attempt to correlate bombesin binding results with mRNA levels, RT-competitive PCR was performed using total RNA preparations of the same cell lines (Figure 3). As expected in competitive PCR, the upper signal (GRP-R mRNA) progressively increased as the lower signal (competitor) decreased. The estimated GRP-R mRNA level (amol/0.25 µg total RNA) was: LoVo E2, 25×10^{4}; LoVo C5, 8×10^{3}; Caco 2, 1400. Differences between two separate determinations of the equivalence point never exceeded one 3-fold dilution of the competitor. Thus, in these colorectal cancer cell lines, GRP-R mRNA levels, as determined by RT-competitive PCR, were in agreement with the results of binding studies.

GRP-R mRNA in normal colonic tissue and tumours

To test whether GRP-R mRNA was detectable in normal colonic tissue, total RNA was isolated separately from colonic epithelium, submucosa and muscle. Integrity of the lamina

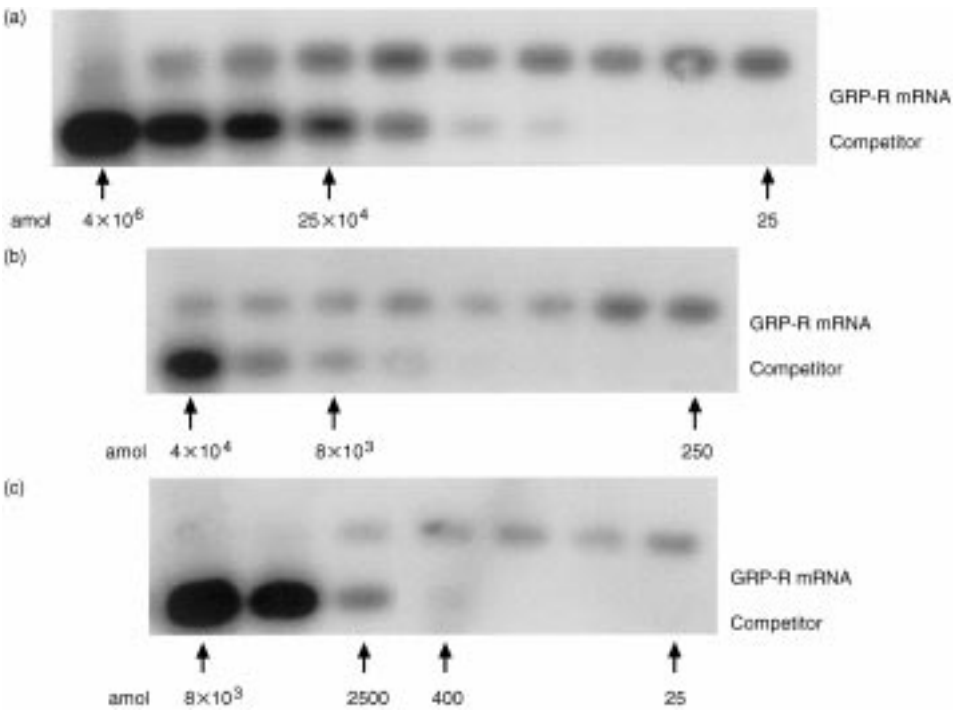

Figure 3. Reverse transcription (RT)-competitive polymerase chain reaction (PCR) amplification of gastrin releasing peptide receptor (GRP-R) mRNA in LoVo E2 (a), LoVo C5 (b) and Caco-2 (c) cell lines. Blotted PCR products were hybridised to oligonucleotide D, to visualise the two fragments (584 and 470 bp). Different amounts of competitor in reaction tubes are indicated (amol). Equal intensity of the hybridisation signals represents equal starting amounts of GRP-R cDNA and competitor in the PCR. Representative of at least five experiments.

propria on histological control sections confirmed that the first fraction contained only epithelial cells (Figure 4). Figure 5 shows representative results of RT–PCR amplification of the GRP-R mRNA and hybridisation of PCR products in epithelial and muscle RNA fractions. A strong GRP-R signal was observed in all muscle specimens, but there was no signal in epithelial specimens. A faint signal was observed in one out of five submucosal extracts (not shown). In parallel, a 838 bp β-actin signal was detected after RT–PCR in all samples (Figure 5): this confirmed the efficiency of the RT reaction.

GRP-R mRNA levels were then estimated in tumour specimens using RT-competitive PCR (Table 1). The GRP-R receptor 584 bp sequence was observed after PCR amplification in 27 of 29 (93%) tumour samples. The absence of contaminating normal mucosa or muscle was verified on adjacent tumour sections (not shown). The estimated GRP-R mRNA level varied from 8000 to 52 amol in positive samples. Figure 6 shows the results of RT-competitive PCR in two representative tumours with a 2-log difference in GRP-R mRNA level. Considering the possible neuroendocrine differentiation of colon cancer [19], tumour sections were stained with chromogranin A antibody. Only five of 29 tumours showed chromogranin A positive cells, with a density that never exceeded 1% of tumour cells and no clustering (not shown).

GRP-R mRNA levels are related to dedifferentiation and lymphatic vessel invasion

The estimates of GRP-R mRNA levels were tentatively correlated with the main characteristics of the 29 colorectal tumours. Table 1 shows these characteristics, including TNM staging, the site of the primary tumour and the grade of differentiation. In univariate analysis, GRP-R mRNA levels did not differ according to tumour site. Considering tumour

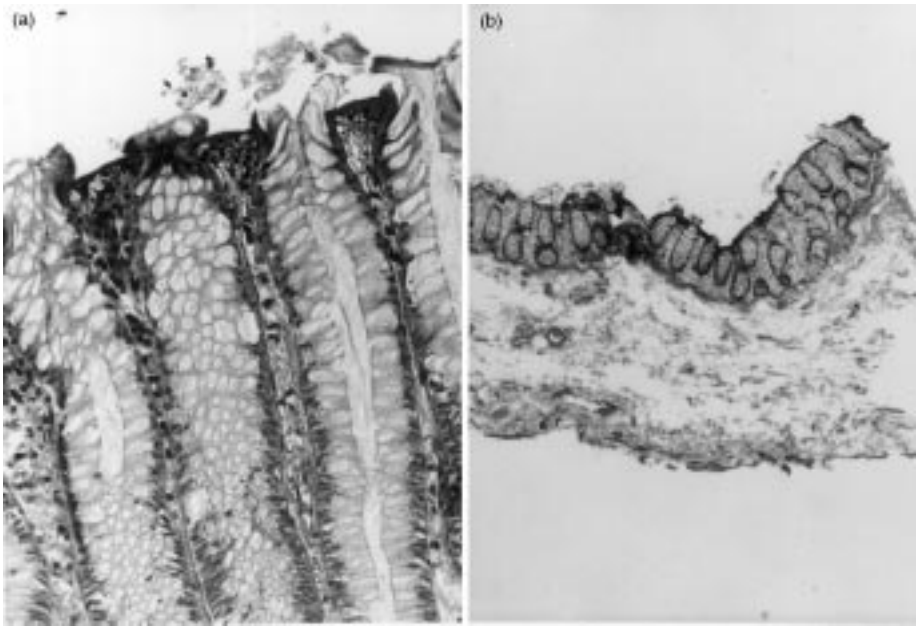

Figure 4. Illustrative example of submucosa plus mucosa fragment. (a) High-power view of the mucosal residue: lysis of superficial epithelium with respect to the lamina propria (haematoxylin and eosin, ×160). (b) Low-power view, showing the absence of residual muscle tissue (haematoxylin and eosin, ×60).

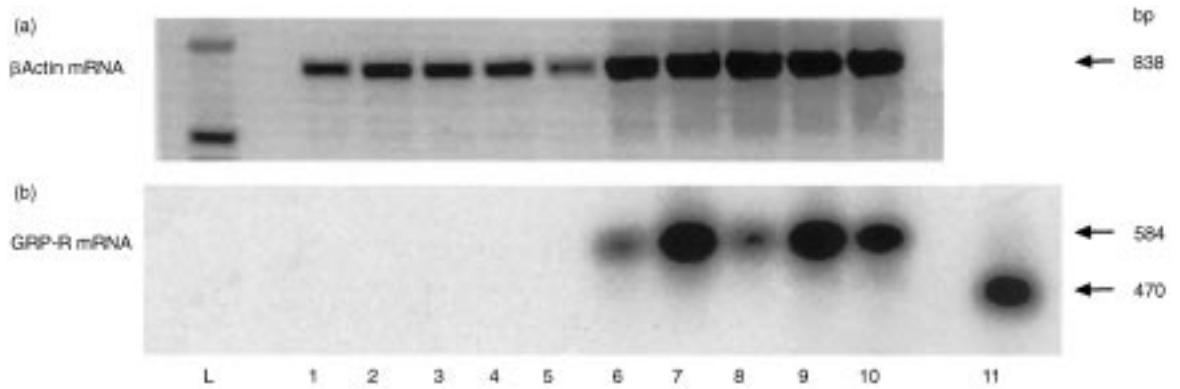

Figure 5. Reverse transcription–polymerase chain reaction (RT–PCR) amplification of β-actin and gastrin releasing peptide receptor (GRP-R) mRNAs in normal colonic wall. (a) RT–PCR amplification of β-actin mRNA from comparable RNA amounts in specimens of epithelium (lanes 1–5) and colonic smooth muscle (lanes 6–10) (ethidium bromide staining). As previously described, β-actin mRNA is overexpressed in muscle compared with epithelial extracts [14]. (b) RT–PCR amplification of GRP-R mRNA and oligonucleotide hybridisation in the same samples as (a) Lane 11, competitor; lane L, 1 kb DNA ladder. RT– in (a) and (b) was performed simultaneously.

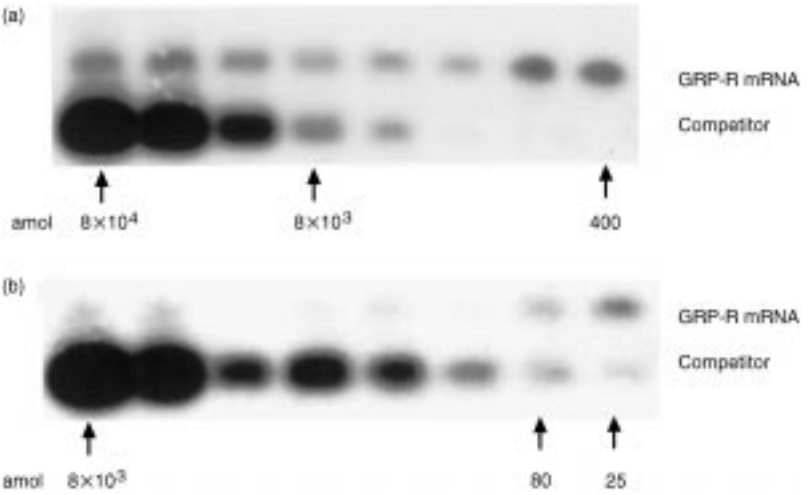

Figure 6. Reverse transcription (RT)-competitive polymerase chain reaction (PCR) amplification of gastrin releasing peptide receptor (GRP-R) mRNA in two representative colorectal tumours. Hybridisation of the PCR products shows the two 584 and 470 bp fragments. Different amounts of competitor in reaction tubes are indicated (amol). Equal intensity of the hybridisation signals represents equal starting amounts of GRP-R cDNA and competitor in the PCR reaction (tumour A, 8×10^3 amol; tumour B, 80 amol). Representative of five (a) and two (b) experiments.

histology, there was no correlation between GRP-R mRNA levels and the depth of invasion, and the presence (versus absence) of positive nodes or distant metastases (not shown). In contrast, GRP-R mRNA levels were higher in tumours with low or moderate differentiation (grade II–III median 2500 amol, range 165–8000) than in well differentiated tumours (grade I: median 287 amol, range 0–4000; $P < 0.05$). GRP-R mRNA levels were also higher in 11/29 (38%) tumours with lymphatic vessel invasion (median 3250 amol, range 0–8000) (Figure 7) than in the 18 (62%) tumours devoid of lymphatic vessel invasion (median 250 amol, range 0–4000, $P < 0.01$). Multivariate analysis showed that histological grade II–III was correlated to the presence of lymphatic vessel invasion.

Considering tumour characteristics apart from histology, GRP-R mRNA levels were not correlated with p53 accumulation (median p53 index in tumours 28.4%, range 1–98.2%), or with the labelling index (mean labelling index 57.3%, range 21–94%) in univariate analysis.

Table 1. *Gastrin releasing peptide receptor (GRP-R) mRNA levels and characteristics of the* 29 *colorectal tumours. GRP mRNA levels were determined using reverse transcription competitive polymerase chain reaction and expressed in amol*

Tumour	GRP-R (amol)	TNM staging	Histological grade*	Site†
1	0	T2 No Mo	I	L
2	0	T3 N+Mo	III	T
3	52	T3 N+M+	I	R
4	52	T3 No Mo	I	L
5	52	T3 N+Mo	I	R
6	80	T1 No Mo	I	L
7	122	T3 No Mo	I	R
8	165	T1 No Mo	I	L
9	165	T3 No Mo	III	R
10	250	T2 No Mo	I	L
11	250	T2 No Mo	I	L
12	250	T3 N+M+	I	R
13	325	T2 No Mo	I	L
14	325	T2 No Mo	III	L
15	400	T4 N+Mo	I	R
16	580	T3 N+Mo	III	R
17	600	T3 No Mo	I	R
18	800	T3 N+M+	I	L
19	800	T3 No Mo	I	T
20	2500	T3 N+Mo	III	L
21	2500	T3 N+M+	I	R
22	3250	T2 No Mo	I	L
23	3250	T3 No Mo	II	R
24	3250	T3 N+M+	I	L
25	3250	T4 N+Mo	I	L
26	4000	T3 No Mo	I	T
27	6000	T3 N+M+	III	R
28	6000	T3 N+M+	II	L
29	8000	T3 No Mo	II	L

*I, well; II, moderately, III, poorly differentiated. †L, left; T, transverse; R, right colon.

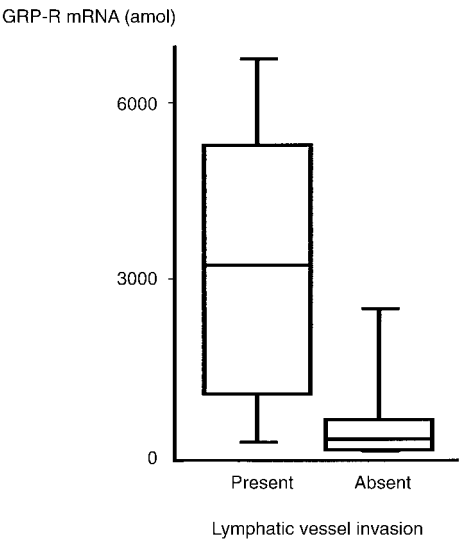

Figure 7. Gastrin releasing peptide receptor (GRP-R) mRNA levels (median, centiles), as estimated by reverse transcription (RT)–competitive polymerase chain reaction (PCR) in the 11 colorectal tumours with lymphatic vessel invasion (median 3250 amol, range 0–8000), and in the 18 without lymphatic vessel invasion (median 250 amol, range 0–4000, $P<0.01$).

DISCUSSION

In the present study, based on RT-competitive PCR, we observed a high frequency of GRP-R expression in human colorectal carcinomas. High GRP-R mRNA levels were correlated with the presence of lymphatic vessel invasion and poor differentiation of the tumours. During the validation step, in agreement with previous RT–PCR and ligand binding studies [11, 20], we could detect GRP-R mRNA in colonic smooth muscle, but not in normal colonic epithelium.

Expression of the GRP-R, as assessed in binding studies, has been previously demonstrated in 30% of colon cancer cell lines, including the LoVo cell line [10, 21], with a receptor density comparable to that presently observed in LoVo E2 cells. In contrast, detection of GRP-R mRNA in cancer cell lines has often been negative in Northern blot analysis, so that more sensitive methods, such as ribonuclease protection assay [8] or RT-competitive PCR amplification were required. The present PCR method was specific, as proven by direct sequencing of the PCR product, and yielded reproducible results. Competitive PCR has indeed been shown to overcome the variability of simple PCR amplification [15]. Interestingly, the decreasing order of GRP-R expression in the studied cell lines was similar by binding and RT-competitive PCR techniques. Although the intracellular mechanisms that regulate expression of the GRP-R at the cell membrane are unknown, this result suggests that regulation is driven, at least partly, at the RNA level. Binding studies in Caco-2 cells were negative, which may reflect the observed low RNA level, but also additional restraints on protein expression or receptor addressing. Further *in vitro* studies are warranted to clarify these points.

Considering colorectal tumours, bombesin/GRP receptors have been previously detected in membrane preparations, through binding studies, with a prevalence of 24–40% [11, 22]. At the mRNA level, GRP-R expression here was much more frequent than suspected in colorectal cancers. This clearly results from the sensitivity gain over binding experiments. Nevertheless, mRNA levels in the present series of tumours were not uniform and practically extended over two orders of magnitude. Confounding factors that could account for these variations were excluded, especially the sampling of necrotic areas, and the possible neuroendocrine differentiation of colorectal tumours [19]. Further studies are needed to determine whether low RNA levels directly explain the lack of bombesin binding in more than 70% of colorectal tumours [11]. Because of the requirement of tumour staging, the size of tumour aliquots available here was too small for analysis of GRP-R at the protein level and multiple sampling of each tumour could not be performed. However, previous studies (some using *in situ* hybridisation) have not suggested that neuropeptide receptor expression differs in separate areas of the same colorectal tumour [11, 23].

The quasi-constant expression of GRP-R mRNA in tumours might simply reflect global deregulation of gene expression, with little or no impact on cell biology. We observed no relationship between mRNA levels and Ki-67 index. Data on direct growth stimulation effects of bombesin in colorectal tumour cell lines are limited, in contrast to other cell types such as small cell lung or pancreatic tumour cells [1, 2, 4, 21]. Moreover, in rats bearing azoxymethane-induced colorectal tumours, bombesin did not stimulate tumour cell proliferation but increased regional (peritoneal) tumour spreading [6]. Thus, stimulation of the bombesin/GRP receptor in colorectal tumours may result in biological effects different from growth stimulation. Further studies using cancer cell lines or tumour models are required to test whether bombesin may enhance the escape of tumour cells via the lymphatic vessels. Interestingly, it was recently demonstrated that ectopic expression of wild-type GRP-R in colonocytes resulted in constitutive activation of the receptor [24]. Whether such a constitutive activation takes place in colorectal tumours expressing this receptor remains to be proved.

The prognostic significance of peptide receptor expression in colorectal cancer has not been thoroughly assessed and appears to depend on the given peptide [23, 25]. The current list of prognostic factors does not include any peptide receptors. Depth of tumour invasion and lymph node status are two major criteria [17]. However, tumour staging may reflect the delay to diagnosis rather than the intrinsic invasiveness of tumour cells. Other factors have been tested, including p53 mutation and proliferation index. Both yielded somewhat controversial results possibly because heterogeneous immunohistochemical methods were used [26, 27]. Two other factors, histological grading and lymphatic vessel invasion, are of prognostic value independently of TNM staging [17, 18]. Interestingly, GRP-R mRNA levels appeared to be significantly correlated to these two factors in the present study. This suggests that GRP-R expression in colorectal carcinomas may have some prognostic value, even though opposite conclusions were reached in a former study based on binding experiments [11].

Abnormal expression of neuropeptide receptor genes in tumours has been described for other peptides, such as somatostatin [23, 28]. Interestingly, the presence of neuropeptide receptors in human tumours has potential clinical implications in tumour treatment and prognosis [29]. Further studies are warranted to determine the pattern of the multiple neuropeptide receptor gene expression in colorectal tumours, and its potential link with the expression of oncogenes and tumour suppressor genes. The RT-competitive PCR strategy used here appears valuable for such studies, because it is sensitive and not tissue consuming.

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Acknowledgements—We wish to thank Dr J.F. Battey and Dr M. Cordier-Bussat for their most useful comments, and Dr Adeleine for his help concerning the statistical analyses. This work was supported in part by a grant from the Programme Hospitalier de Recherche Clinique no. CA-3359.