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NEUROTENSIN RECEPTOR AND ITS mRNA ARE EXPRESSED IN MANY HUMAN COLON CANCER CELL LINES BUT NOT IN NORMAL COLONIC EPITHELIUM : BINDING STUDIES AND RT-PCR EXPERIMENTS

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Neurotensin receptor expression was studied in19 human colon cancer cell lines and normal human colon by i) binding experiments using [^{125}I-Tyr^{3}]-neurotensin; ii) RT-PCR analysis. The following data were obtained: 1) A single class of receptor (Kd ranging from 0.23 to 1. 21 nM) was found in 9 out of 19 cell lines but not in normal colonic epithelium; 2) The Bmax was in the range between 1000 and 85 fmoles / mg protein with SW48 > WiDR > Cl 19A > HCT116 > SW480 > SW620 > Cl 16E > Cl 27H > HT-29. No specific binding was measurable in Caco-2, FRI, CBS, EB, HCT-8, 320HRS, 320DM and LS174T cell lines; 3) A single RT-PCR product was observed in HT-29, SW48, WIDR, Cl 19A, SW480, Cl 16E, Cl 27H, SW620 and HCT116, but not in other cell lines or in normal human colon. It is concluded that the expression of neurotensin receptors in human colon cancer cells is regulated at the mRNA level and occurs upon malignancy in > 40% of colon cancer cell lines. © 1994 Academic Press, Inc.

Neurotensin is localized mainly to the central nervous system and discrete enteroendocrine N cells of the distal gut, especially the ileum (1). It is encoded by a precursor protein which also contains neuromedin N, a structurally related peptide (2). Neurotensin has a number of pharmacological properties in the digestive tract: effects on vascular smooth muscles and gastrointestinal motility, inhibition of gastric acid secretion, stimulation of intestinal, pancreatic and biliary secretions (1). Moreover, neurotensin is trophic for numerous gut tissues including small bowel, pancreas and gastric antrum, and also for liver (3,4).

The neurotensin receptor belongs to the superfamily of G protein-coupled receptors and triggers phospholipase C activation in most target tissues (5). This is in consonance with the fact that intracellular Ca^{2+} appears to be the major mediator of neurotensin action (5). A human neurotensin receptor has been recently cloned (6).

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In keeping with the fact that stimulatory effects of neurotensin on water and electrolyte secretion in intestine are assumed to be neuronally mediated (1,7), we did not observe any neurotensin binding site in epithelial cells from small intestine or colon (author's unpublished results). In contrast, a typical neurotensin receptor has been characterized in the human colon adenocarcinoma cell line HT-29 (8) and the mucus-secreting clone Cl 16E originating from HT-29 cells (9). Moreover, this receptor is functional since it mediates stimulation of phosphatidylinositol turnover (10), cytosolic Ca^{2+} level (9,11) and mucin output (9).

In this context, it is possible to suggest that the neurotensin receptor in HT-29 cells and derived clones, represents an unmasked ectopic expression of receptors due to malignant transformation (7, 12). The purpose of this work was to determine whether the expression of neurotensin receptors is restricted to the HT-29 cell line or whether it is a more general characteristic of human colon cancer cells. Therefore, we have screened 19 human colon cancer cell lines for expression of neurotensin receptors by binding experiments using [^{125}I]-neurotensin and by developing RT-PCR using specific human receptor primers.

Materials and Methods

Cell lines and culture methods - The main characteristics of the human colon cancer cell lines used in this study are described elsewhere [see ref. 12 for an extensive review]. Three stably differentiated clonal derivatives of the HT-29 cell line were also used in this study, i.e. Cl 19A, a chloride-secreting cell line (13); Cl 16E, a mucus-secreting cell line (13); Cl 27H, a cell line differentiated into both mucous and enterocytic cells (14). The cells were routinely grown in plastic flasks with fetal bovine serum as recommended by the American Type Culture Collection (15). All cell lines were harvested in the post-stationary growth phase as described (9).

Collection of intestine and isolation of epithelial cells - Fresh human intestines were collected with the assistance of France-Transplant. They were obtained from cadavers immediately after collection of the organs to be transplanted. Epithelial cells from jejunum and colon were isolated using EDTA as described (16).

Binding of [^{125}I]-neurotensin to cell membranes - Membranes were prepared from cultured or isolated cells as described (9,16). The binding assay was carried out as reported (9). Briefly, membranes (100 µg/ml) were incubated for 30 minutes at room temperature with [^{125}I-Tyr^3]-labeled neurotensin (0.04 nM) and increasing concentrations of unlabeled neurotensin or neuromedin N, in 20 mM HEPES (pH 7.5) containing 2% (w/v) BSA and 0.1% (w/v) bacitracin. The reaction was stopped and the radioactivity bound to membranes was measured. Specific binding was calculated as the difference between the amount of radioactivity bound in the absence or presence of 1 µM unlabeled neurotensin. All binding data were analyzed using the LIGAND computer program (Elsevier-Biosoft) developed by Munson and Rodbard (17).

RT-PCR experiments - Total RNA was extracted from cells by the RNazol method according to the protocol suggested by the supplier (Bioprobe Systems). Ten µg of total RNA were reverse transcribed and 5 µl of the resulting cDNA mixture were submitted to PCR using specific primers. For the human neurotensin receptor (6), sens H544 (5'-CGAAGCCGCACCAAGAAGTT-3') and antisens H1067 (5'-AGGATGGGGTTGATGGTGGAG-C-3') primers were used for 30 cycles (denaturated at 94°C, one minute; annealed at 57°C, one minute; extended at 72°C, one minute). For the human VIP receptor (18), sens VR622 (5'-GGGCTCGGTGGGCTGTAAGG-3') and antisens VR14 (5'-GACCAGGGAGACTTCGGCT-TG-3') primers were used for 25 cycles (denaturated at 94°C, one minute; annealed at

58°C, one minute; extended at 72°C, one minute). For glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), taken as a control for a house-keeping protein, sens 5'-TGAAGGTCGGAGTCA-ACGGATTTGGT-3' and antisens 5'-CATGTGGGCCATGAGGTCCAC-CAC-3' were used for 25 cycles as described above for the VIP receptor. PCR amplification mixtures were then analyzed on 1% agarose gel. After ethidium bromide staining, DNA bands were visualized with an ultraviolet transilluminator. They were subsequently submitted to Southern hybridization with the [^{32}P]-labeled human neurotensin receptor cDNA fragment 544-919 (6), full-length human VIP receptor cDNA probe hIVR8 (18) or GAPDH probe (Clontech). When total RNA had been incubated with RNAse A (DNAse free) prior to PCR , no PCR product was observed whatever the couple of primers, ruling out the contamination by genomic DNA in our experiments.

Results and Discussion

Nineteen human colon cancer cell lines were first screened by classical ligand binding assay using [^{125}I]-neurotensin as a tracer. Figure 1 (left) shows Scatchard plots obtained with three positive cell lines, i.e. HT-29, Cl 16E and SW480. Linear plots were observed that were compatible with the existence of one population of high affinity receptors. The same was true for SW48, WiDR, Cl 19A, SW620 , Cl 27H and HCT116 cell lines (not shown). Table I shows the dissociation constant (Kd) and binding capacity (Bmax) measured in the 9 positive cell lines. The Kd were very similar ranging from 0.23 to 1. 21 nM, while the Bmax were quite different ranging from 85 to 1000 fmol/mg protein. In all these cell lines (not shown), including SW480 (Figure 1, right), neuromedin N, a neurotensin-related peptide (2), competed with [^{125}I]-neurotensin for binding to cell membranes. The ratio of Ki for neuromedin N / ki for neurotensin was close to 20 whatever the cell line, assessing for the expression of a typical neurotensin-preferring receptor in human colon cancer cells. No specific [^{125}I]-neurotensin binding could be observed in 10 other human colon adenocarcinoma cell lines, including Caco-2, Ls174T, COLO 320DM, COLO 320HRS, COLO 205, HCT8, ALA, CBS, EB and FRI. Nor was there any specific binding in membranes prepared from epithelial cells isolated from normal human

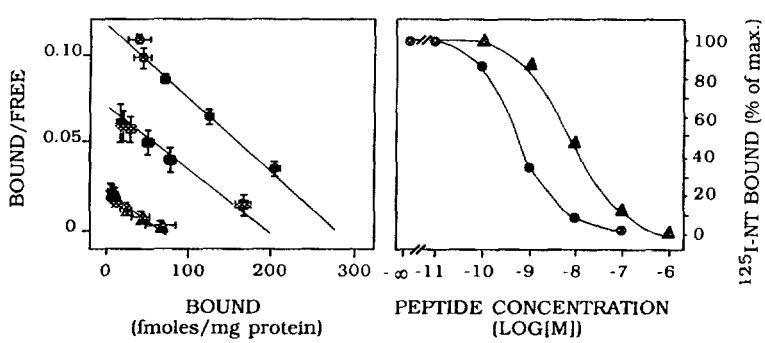

FIG. 1. **Neurotensin binding to human colon cancer cell lines.** Left, Scatchard analysis of neurotensin binding to membranes prepared from HT-29 (▲), Cl 16E (■) or SW 480 (●) cells. Right, inhibition of [^{125}I]-neurotensin binding to SW 480 cell membranes by increasing concentrations of unlabeled neurotensin (●) or neuromedin N (▲). Each point is the mean of three determinations.

TABLE 1. *Quantitative analysis of neurotensin receptors in human colon cancer cell lines*

Cell lines	Kd (nM)	Bmax (fmoles/mg protein)
SW48	0.99 ± 0.08§	1000 ± 18
WiDR	1.21 ± 0.04	525 ± 4
Cl 19A	0.23 ± 0.03	434 ± 48
HCT116	0.80 ± 0.23	311 ± 87
SW480	0.43 ± 0.04	294 ± 56
SW620	0.80 ± 0.05	279 ±4
CL 16E	0.45 ±0.07	208 ± 41
Cl 27H	0.71 ± 0.10	177 ± 50
HT-29	0.69 ± 0.10	85 ± 28

§ *Mean ± SEM; n=3*

colon or jejunum (not shown). From this first series of data, it can be concluded that 1) neurotensin receptors are expressed in 9 out of 19 human colon cancer cell lines, i.e. 47%; 2) this expression is not detectable in normal colon or small intestine.

To further characterize the expression of neurotensin receptors in human colon cancer cells, RT-PCR analysis of neurotensin receptor mRNA was developed by using human neurotensin receptor oligonucleotide primers (see Materials and Methods). A single PCR product of the expected size (523 bp) was observed in HT-29, Cl 16E, Cl 19A, Cl 27H, SW48, SW480, SW620, WiDr and HCT116 cell lines (Figure 2).The PCR product amplified from Cl 16E cells was shown to be 100% homologous to the corresponding sequence in the human neurotensin receptor cDNA (6). In contrast, no PCR product could be amplified in

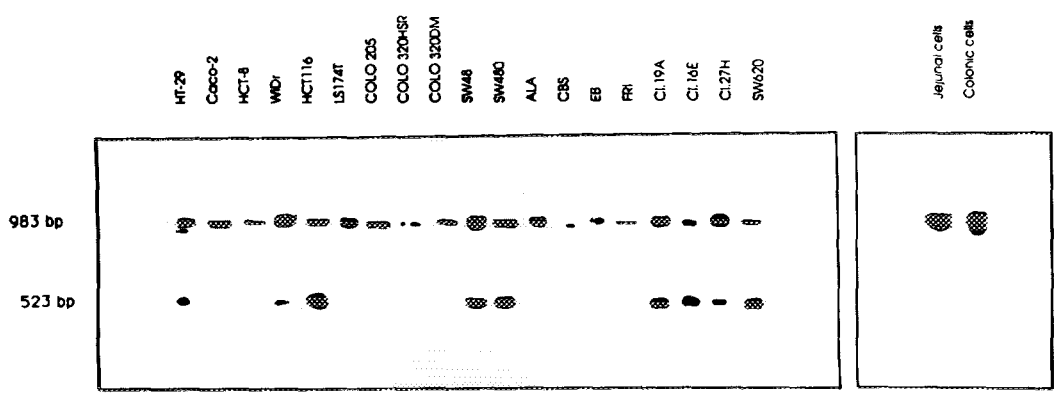

FIG. 2. **PCR-based detection of mRNA expression of neurotensin receptor in human colon cancer cell lines and normal human intestine.** *cDNAs from 19 human colon cancer cell lines and epithelial cells isolated from normal human small intestine or colon were used as templates for PCR reactions using specific primers for human neurotensin receptor or GAPDH (see Materials and Methods). Southern hybridizations with specific[^{32}P]-labeled probes are shown.*

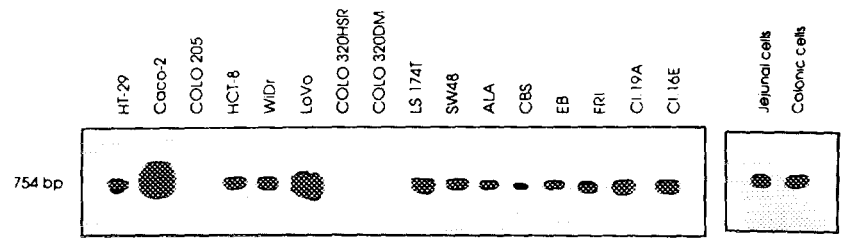

FIG. 3. **PCR-based detection of mRNA expression of VIP receptor in human colon cancer cell lines and normal human intestine.** cDNAs from human colon cancer cell lines and epithelial cells isolated from normal human small intestine or colon were used as templates for PCR reactions using specific primers for human VIP receptor (see Materials and Methods). Southern hybridization with a specific[^{32}P]-labeled probe is shown.

all other cell lines tested (Figure3). The expected PCR product for GAPDH (983 bp), taken as a control, was observed in all cell lines. Therefore, a perfect correlation is noted between binding studies (Table 1) and RT-PCR experiments (Figure2), supporting the fact that the expression of neurotensin receptors in human colon cancer cells is regulated at the mRNA level. RT-PCR experiments further confirmed the absence of neurotensin receptors in normal epithelial cells from human colon or small intestine since no PCR product could be detected in these two intestinal segments (Figure 2). This contrasts with the expression of vasoactive intestinal peptide (VIP) receptors which occurs in both normal intestinal epithelial cells from colon or small intestine, and in most human colon cancer cell lines (7, 12, 19, 20). This was confirmed here by RT-PCR experiments (Figure 3) using specific primers for the human intestinal VIP receptor (see Materials and Methods). Indeed, the expected PCR product (754 bp) was observed in normal tissues and in all cell lines tested but COLO cell lines (Figure 3).

These data strongly support the idea that the expression of neurotensin receptors in human colon cancer cell lines is ectopic and frequent, e. g. in 47% of the cell lines tested. However, considering the fact that HT-29 cells and the three clones Cl 16E, Cl 19A and Cl 27H (13, 14) originate from the same patient, the frequency of neurotensin receptor expression in colon cancers could be somewhat lower than 47%. We should subsequently investigate resected colonic tumors in order to give the exact frequency of neurotensin receptor expression in colon cancers. It can be anticipated that the herein described RT-PCR assay for neurotensin receptor mRNA should be very helpful to achieve this goal. It may be hypothesized that expression of neurotensin receptors in colon cancers represents a de-repression and a reversion to a fetal phenotype as shown previously for other proteins such as sucrase isomaltase or dipeptidylpeptidase IV (reviewed in 12). This contrasts with the expression of the VIP receptor in colon cancers which can be considered as a normal phenotype simply conserved during malignant transformation of the colon (7, 12, 19, 20). What can be learnt also from this study is that neurotensin receptor expression is not correlated with any classical differentiation phenotypes in the colon cancer cell lines.

Indeed, the neurotensin receptor and its mRNA are detected in undifferentiated cells, e.g. for example HT-29 or HCT 116, and also in cells which have undergone enterocyte-like differentiation , e.g. Cl 19A or goblet cell-like differentiation, e.g. Cl 16E (13). Conversely, neurotensin receptors are not significantly expressed in Caco-2 cells which show typical enterocyte differentiation at confluency (12).

Our data raise the question of the biological significance of neurotensin receptors in human colon cancer cells. It is worth pointing out that neurotensin stimulates phospholipase C in colon cancer cells (5), an effect which may lead to regulation of cell growth through subsequent activation of protein kinase C. In this context, it is of interest to notice that neurotensin does stimulate the growth in vivo of the human colon cancer cells LoVo xenografted in nude mice (21) and enhances chemical carcinogenesis in rat colon (22). Moreover, neurotensin and its mRNA have been characterized in certain human colon cancer cell lines (21). This includes HT-29 cells which are also equipped with neurotensin receptors, suggesting a possible autocrine regulation of colon cancer cells by neurotensin. Such an autocrine role of neurotensin has been recently demonstrated in human prostate cancer (23).

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