# Neurotensin Expression and Release in Human Colon Cancers

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**Neurotensin (NT), a distal gut peptide released by intraluminal fats, is trophic for normal small bowel and colonic mucosa. In addition, NT stimulates growth of certain colon cancers; the mechanism for this effect is not known. The purpose of this study was to determine whether human colon cancers (HCC) (1) express the mRNA for NT/neuromedin N (N), (2) produce NT peptide, and (3) express the mRNA for a functional NT receptor (NTR). RNA was extracted from four HCC cell lines in culture, nine HCC lines established in athymic nude mice, and from six HCC and adjacent normal mucosa from freshly resected operative specimens; the RNA was analyzed for NT/N mRNA by Northern hybridization with a complementary DNA probe. Neurotensin peptide content, NTR expression, and intracellular $Ca^{++}$ ($[Ca^{++}]_i$) mobilization in response to NT were evaluated in three HCC cell lines (LoVo, HT29, HCT116). Neurotensin/N mRNA transcripts were identified in all four of the HCC cell lines and in one of nine HCC in nude mice. Neurotensin expression was found in two of six freshly resected HCC and in none of the six corresponding samples of normal mucosa. Neurotensin peptide was identified by RIA in LoVo, HT29, and HCT116. In addition, NTR mRNA was found in HT29 and HCT116. Neurotensin stimulated $[Ca^{++}]_i$ mobilization in HCT116 (without serum) and in LoVo (with 0.25% serum). These findings demonstrate the presence of NT/N mRNA and NT peptide and the presence of a functional NTR in certain HCC. Neurotensin, a potent trophic factor for normal gut mucosa, may function as an autocrine growth factor in certain human colon cancers.**

COLON CANCER IS the second-leading cause of cancer death in the United States, with a yearly incidence of approximately 150,000 new cases.[1] Significant advances have been made in the treatment of colon cancer; however, the overall 5-year survival has remained the same for the past 50 years.[2] The specific intracellular mechanisms responsible for growth of colon cancers have not been clearly discerned. Gastrointestinal hormones that are trophic for normal gut mucosa may affect growth of some cancers that arise from the alimentary tract.[3] Receptors for gastrointestinal hormones have been identified on human colon cancers and, in fact, a number of reports have demonstrated that these hormones can modulate (either increase or decrease) growth by mechanisms involving endocrine, paracrine, or autocrine effects.[3-7]

Neurotensin (NT), a tridecapeptide localized mainly to the central nervous system and discrete enteroendocrine cells (N cells) of the distal small bowel mucosa,[8,9] is released from the gut by intraluminal fats.[10] The structure of the gene encoding NT has recently been determined by Dobner and colleagues.[11,12] In all species, a precursor protein of 169–170 amino-acid residues encodes both NT and neuromedin N (N), a structurally related six-amino-acid peptide. Neurotensin stimulates proliferation of pancreas and small bowel and colonic mucosa.[13-16] In addition to these trophic effects in normal organs, NT promotes carcinogenesis in the stomach and colon of rats,[17,18] enhances the growth of mouse (MC-26) and human (LoVo) colon cancers *in vivo*,[19] and stimulates growth of a human pancreatic cancer (MIA-Paca2) *in vitro*.[20] Neurotensin is also present in approximately 50% to 60% of all human small cell lung cancers and stimulates growth of certain of these cells in an autocrine fashion.[21,22] Collectively, these data suggest an important role for NT in the growth of both normal and neoplastic tissues.

The purpose of this study was to evaluate our hypothesis

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that NT may act to stimulate the growth of various colon cancers by an autocrine mechanism. To prove an autocrine hypothesis, several criteria must first be met: (1) determination of whether human colon cancers express NT/N mRNA, (2) determination of whether NT peptide is present and released by stimulation, (3) determination of whether colon cancers express mRNA for the NT receptor (NTR), and (4) determination of whether these receptors are linked to a signal transduction pathway, for example, mobilization of intracellular calcium ($[Ca^{++}]_i$).

## Materials and Methods

### *Cell Culture*

Four human colon cancer cell lines were examined. LoVo and HT29 were obtained from the American Type Culture Collection (Rockville, MD), and the HCT116 and CBS colon cancer cell lines were obtained from Dr. Michael Brattain (Bristol-Baylor Laboratory, Houston, TX). Characterization of these lines has been previously described in detail.[23-25] LoVo (passage 25–35) and HT29 (passage 25–35) are maintained in Dulbecco's modified Eagles medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT). HCT116 (passage 5–10) and CBS (passage 3–7) are maintained in McCoy's medium 5A (Gibco) and 10% FBS.

All cells are grown in a humidified atmosphere of 95% air and 5% $CO_2$ at 37 C. Cells are routinely passed by removing the medium and overlaying the cell monolayer with 0.25% trypsin: 0.1% ethylenediaminetetraacetic acid (EDTA) mixture. Cell cultures are monitored for mycoplasma contamination; no mycoplasma growth has been detected.

### *RNA Isolation, Northern Blot Analysis*

RNA was isolated from four cell lines (LoVo, HT29, HCT116, and CBS) when approximately 100% confluent in T150 culture flasks. Briefly, the cell monolayer was washed twice with cold, sterile phosphate-buffered saline (PBS) and then RNA extracted by the method of Schwab and co-workers.[26] In addition, nine human colon cancers that had previously been established as cell lines in male athymic nude mice (BALB/c; Life Science, St. Petersburg, FL) were removed and RNA extracted. Relevant clinical information regarding tumor and patient characteristics are shown in Table 1. Also, sections of colon cancers and adjacent normal mucosa (5 to 10 cm from the primary site) from operative specimens of six patients were immediately washed with cold phosphate-buffered saline, snap-frozen with liquid nitrogen, and stored at −80 C until RNA extraction. Total RNA was prepared from tumor xenografts and operative specimens using the acid guanidinium isothiocyanate-phenol-chloroform procedure.[27]

Polyadenylated (Poly[A]$^+$) RNA was selected from all samples by oligo(dT) cellulose (type III, Collaborative Research Inc., Bedford, MA) column chromatography. The final RNA concentration was quantified by measuring its absorbance at 260 nm. For Northern blot analyses, poly(A)$^+$ RNA was separated on a 1.2% agarose-formaldehyde gel. Size-fractionated RNA was transferred to nitrocellulose filters (Sartorius, Goettingen, Germany) by capillary action. After transfer, filters were baked for 2 hours in a vacuum oven at 80 C. The canine NT/N complementary DNA (cDNA) probe, provided by Dr. Dobner,[10] contains a 729 bp fragment subcloned into a pGEM4 vector and the rat cyclophilin cDNA probe contains a 680 bp insert into plasmid SP65.[28] $^{32}$P-CTP (New England Nuclear Research Products, Boston, MA) labeled antisense probes were synthesized by an *in vitro* transcription procedure (labeling kit, Promega Corp., Madison, WI). The rat NTR, provided by Dr. Nakanishi,[29] contains a 760 bp *Eco*47III *Sma*I fragment, which was excised from clone prNTR2 and labeled with $^{32}$P-ATP (New England Nuclear) using a random primer kit (Stratagene, La Jolla, CA).

TABLE 1. *Characteristics of Human Colon Cancers Established in Athymic, Nude Mice*

| Cell Line (Passage Extracted) | Colon Primary Site | Xenograft Site | Tumor Differentiation | Patient Age (yr)/Sex |
|---|---|---|---|---|
| KIT (P1) | Cecum | Retroperitoneal metastasis | Well | 58/M |
| RIP (P26) | Transverse | Liver metastasis | Moderate | 61/F |
| FRAN (P7) | Descending | Lymph node metastasis | Moderate | 58/F |
| WIN (P15) | Sigmoid | Peritoneal implant | Moderate | 58/F |
| AIK (P5) | Transverse | Colon cancer | Moderate | 52/F |
| WOOD (P1) | Cecum | Peritoneal implant | Poor | 54/F |
| TAY (P1) | Descending | Colon cancer | Moderate | 68/M |
| MOR (P1) | Transverse | Colon cancer | Moderate | 58/M |
| OLI (P1) | Transverse | Colon cancer | Moderate | 41/M |

The passage numbers used for RNA extraction, primary tumor site, site of tumor used for implantation into mice, tumor differentiation, and patient age and sex are shown.

RNA blots were prehybridized and hybridized at 43 C (NTR) or 65 C (NT/N, cyclophilin) as previously described.[30,31] After hybridization, filters were washed three times in 2× SSC (1 × SSC = 0.15 mol/L sodium chloride, 0.015 mol/L sodium citrate; pH 7.0) with 0.1% sodium dodecyl sulfate (SDS) at room temperature for 5 minutes, then washed two times in either 1× SSC with 0.1% sodium dodecyl sulfate at 43 C (for NTR) or 0.1× SSC with 0.1% sodium dodecyl sulfate at 65 C (for NT/N and cyclophilin). Filters were blotted dry and exposed to x-ray film (XAR-5; Eastman Kodak Co., Rochester, NY) in the presence of double intensifying screens at −80 C.

*NT Release Experiments, NT Radioimmunoassay*

LoVo, HT29, and HCT116 cells were scraped and sonicated for 30 seconds using a sonic dismembranator (Model 300, Fisher Sonicator) for measurements of intracellular NT levels by radioimmunoassay (RIA) expressed as picograms (pg)/3 × $10^5$ cells. In addition, for NT release experiments, LoVo cells (5 × $10^5$) were harvested by trypsin (1:250, Gibco) and plated into 35-mm tissue culture dishes in Dulbecco's modified Eagles medium + 5% FBS. After a 2-day incubation, cells were washed twice with 2 mL oxygenated Krebs-Ringer bicarbonate buffer (KRBB, pH 7.4) containing HEPES (10 mmol/L), bovine serum albumin (0.1%), glucose (2.5 mmol/L), and incubated for a further 30 minutes in fresh Krebs-Ringer bicarbonate buffer containing either 8-bromo-cAMP (0.1 mmol/L) (Sigma Chemical Co., St. Louis, MO), L-arginine (0.1 to 10 mmol/L) (Sigma), or vehicle (control). After this incubation period, media and cells were saved for measurement of NT peptide levels by RIA. Results are calculated as per cent fractional release (amount of NT secreted into the medium × [amount of NT secreted into the medium + amount of NT present in the cell extract]$^{-1}$ × 100) and are expressed as per cent of control.

Measurement of NT levels in incubation media and sonicated cells was carried out using a specific RIA established in our laboratory and previously described in detail.[9] Results are expressed as the mean ± standard error of the mean (SEM) and analyzed using Student's unpaired t test at the 0.05 level of significance.

*Intracellular Calcium ($[Ca^{++}]_i$) Mobilization*

LoVo and HT29 (in Dulbecco's modified Eagles medium + 5% FBS) and HCT116 (in McCoy's 5A + 5% FBS) were grown on 25-mm diameter glass coverslips at 37 C. The coverslips were coated with 25% Matrigel (Collaborative Research) solution to enhance attachment of cells. The cells were incubated for 60 minutes at 25 C with 2 mL Krebs-Ringer Henseleit (KRH) buffer (pH 7.4) containing 10 μmol/L fura-2/acetoxylmethyl ester (Molecular Probes, Inc., Eugene, OR). Loaded cells were washed twice with fresh Krebs-Ringer Henseleit and reincubated in Krebs-Ringer Henseleit with 0.1% bovine serum albumin at 25 C for 30 minutes in the dark before fluorescence microscopy. The cells were placed in an open perfusion microincubator (PDMI-2, Medical System Corp., Greenvale, NY) and stimulated by synthetic NT ($10^{-10}$ mol/L to $10^{-7}$ mol/L) (Peninsula Laboratories, Inc., Belmont, CA) diluted in either 0.25% FBS or normal saline at 37 C. Fura-2 fluorescence intensity was measured using a dual-wavelength excitation spectrofluorometer (Spex Industries, Inc., Edison, NJ). The wavelengths were 340 and 380 nm for excitation. $[Ca^{++}]_i$ was calibrated by lysing cells with 0.03% Triton X-100 and 5 mmol/L ethylene glycol tetra-acetic acid. Concentration of $[Ca^{++}]_i$ was calculated by the method of Grynkiewicz and colleagues[32] using a Kd for calcium as 224 nmol/L.

## Results

*Expression of NT/N mRNA in Human Colon Cancers*

To detect mRNA expression for NT/N in various human colon cancers, we performed Northern analyses (10 to 20 μg poly(A)$^+$ RNA/lane) using a canine NT/N cDNA probe followed by high-stringency posthybridization washes (Fig. 1). Neurotensin/N mRNA expression was found in all four *in vitro* human colon cancer lines. Only one of nine human colon cancer xenografts (KIT) expressed NT/N mRNA. KIT is a well-differentiated adenocarcinoma obtained from a retroperitoneal metastasis of a cecal primary. From resected specimens, NT/N mRNA was expressed in two of six colon cancers and in none of the corresponding samples of adjacent normal mucosa. Both of the resected colon cancers expressing NT/N mRNA were moderately differentiated adenocarcinomas that had spread to regional lymph nodes; one of the primary cancers originated from the cecum and the other from the sigmoid. In all the colon cancers that expressed the NT/N mRNA, the characteristic transcripts of 1.0 and 1.5 kb sizes were demonstrated as previously described for normal gut mucosa with the 1.0 kb transcript the predominant form.[11,31]

*Neurotensin Peptide Is Produced and Released From Human Colon Cancer Cells*

Radioimmunoassay measurement of LoVo, HT29, and HCT116 cells demonstrated the presence of NT peptide in varying amounts with the largest concentration in HCT116 cells (Fig. 2). The ability of LoVo cells to release NT when stimulated with various agents was tested. 8-Bromo-cAMP (0.1 mmol/L) and L-arginine (0.1, 1.0, and

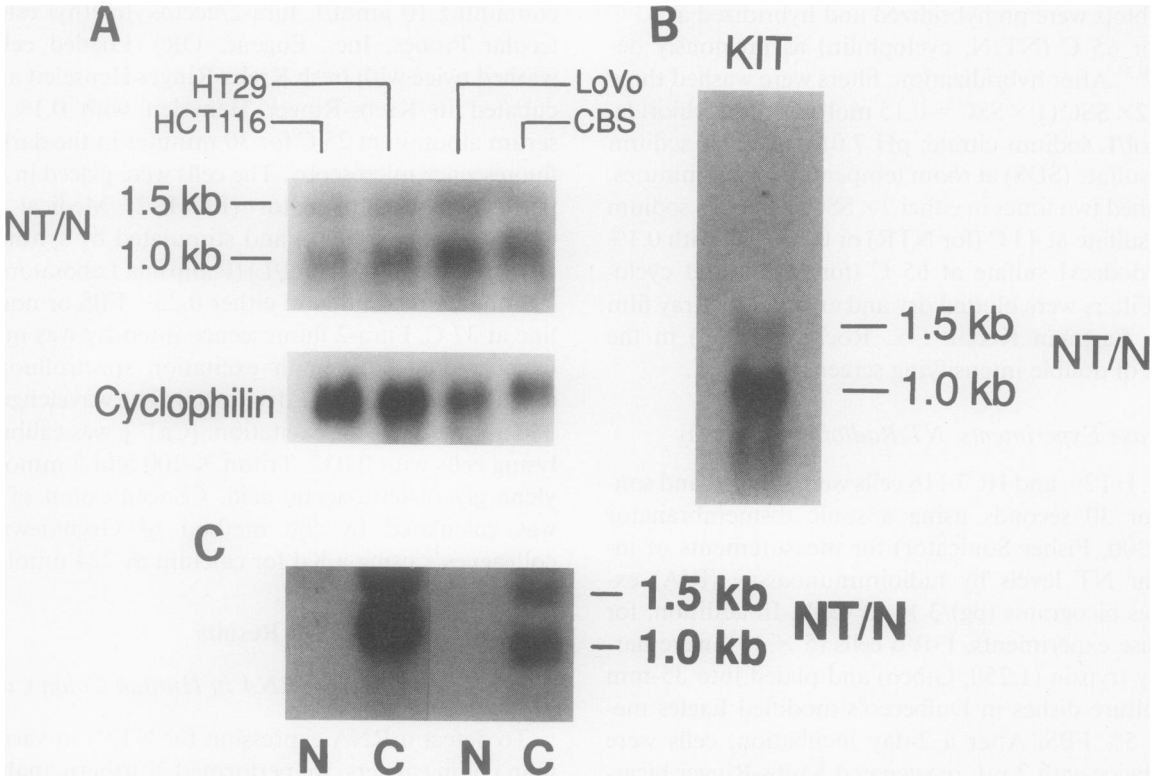


FIG. 1. Expression of NT/N mRNA in human colon cancers: 10–20 μg poly(A)$^+$ RNA from cultured colon cancer cell lines (A), human colon cancer xenografts (B), or colon cancers (C) and adjacent normal mucosa (N) (C) was subjected to Northern blot hybridization with the $^{32}$P-labeled canine NT/N cDNA probe and washed at high stringency. Hybridizing bands were visualized by autoradiography with intensifying screens for 5 to 7 days. The size of the hybridizing RNA species was derived from the migration of the two large ribosomal RNAs as visualized by ethidium bromide staining.

10 mmol/L) were added to the incubation medium. 8-Bromo-cAMP stimulated an approximate fourfold release of NT. L-arginine stimulated NT release in a dose-dependent fashion with the 1- and 10-mmol/L doses significantly elevated compared with control (Fig. 3). Therefore, NT peptide is produced and stored in certain colon cancers and can be released into the medium after stimulation.

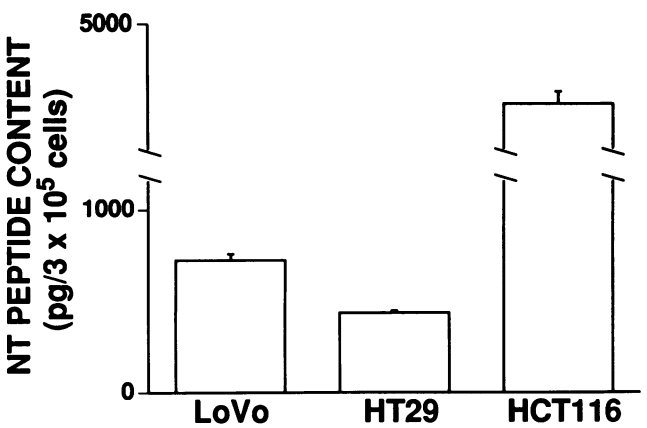


FIG. 2. NT peptide content (pg/3 × 10$^5$ cells) in LoVo, HT29, and HCT116.

*NTR mRNA Is Expressed in Cultured Human Colon Cancers*

To determine expression of NTR mRNA in cultured human colon cancers, Northern blot analysis using a rat NTR cDNA was performed, followed by relatively low-stringency posthybridization washes. We detected a single hybridization band for NTR with an estimated mRNA size of approximately 3.8 kb in HT29 and HCT116 colon cancer lines (Fig. 4). By the methods used in this study, we were unable to identify NTR expression in LoVo (data not shown).

*Neurotensin Stimulates [Ca$^{++}$]$_i$ Mobilization in Colon Cancer Cells*

We next examined whether the addition of NT resulted in mobilization of [Ca$^{++}$]$_i$ in HT29, HCT116, and LoVo cell lines. Binding of NT to its specific receptor has been shown to alter [Ca$^{++}$]$_i$ in other cell lines with functional NTR.[33–36] Similar to the findings of others,[35,36] NT stim-

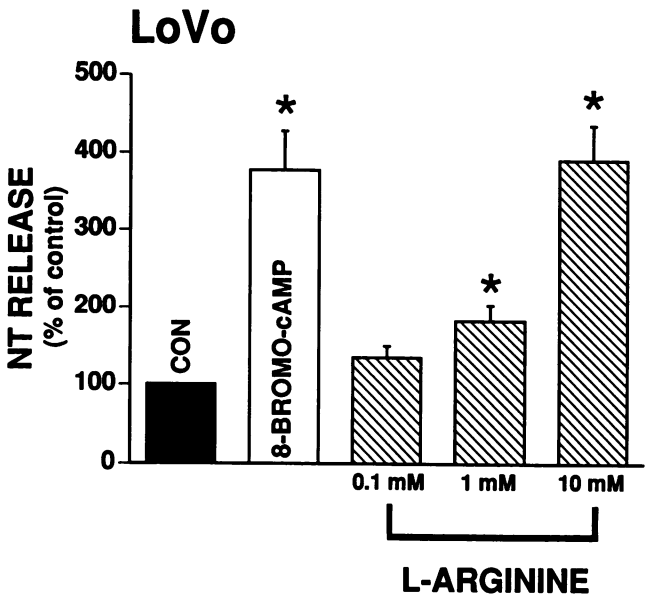


FIG. 3. Effect of 8-bromo-cAMP (0.1 mol/L) and L-arginine (0.1, 1, and 10 mmol/L) on NT release from LoVo cells. Error bars indicate SEM of six plates, and data represent results from three separate experiments. NT fractional release was determined and expressed as a per cent of control. *P < 0.05 *versus* control.

ulated mobilization of $[Ca^{++}]_i$ in the human colon cancer cell line, HT29. Neurotensin ($10^{-7}$ mol/L), in the absence of FBS, caused a rapid, transient increase in $[Ca^{++}]_i$. In addition, NT ($10^{-7}$ mol/L) increased $[Ca^{++}]_i$ in HCT116 cells (Fig. 5).

In the absence of 0.25% FBS, NT did not affect $[Ca^{++}]_i$ levels in LoVo cells; however, in the presence of 0.25% FBS, which by itself did not affect $[Ca^{++}]_i$, NT ($10^{-7}$ mol/L) stimulated $[Ca^{++}]_i$ mobilization (Fig. 6).

## Discussion

The expression of NT/N mRNA was evaluated from various human colon cancers that were maintained as tissue culture lines and as xenografts in nude mice, and from freshly resected operative specimens. All four of the *in vitro* human colon cancer lines, one of nine human colon cancer xenografts, and two of six freshly resected human colon cancers expressed NT/N mRNA. In all colon cancers expressing the NT/N gene, the characteristic transcripts of 1.0 and 1.5 kb sizes, which have previously been demonstrated in normal small bowel mucosa,[11,31] were identified with the 1.0 kb mRNA, the more abundant form. The fact that these colon cancers express the two NT/N mRNAs in the same relative proportion as normal small bowel suggests that the production of these mRNA species are the same in normal and neoplastic gut tissue. Neurotensin/N expression in the gut is mainly localized to the distal small bowel; little appreciable NT/N expression or NT peptide is found in normal colonic mucosa.[31] The reason that some, but not all, colon cancers express NT/N mRNA is not readily apparent from this study; however, we speculate that the degree of tumor differentiation may contribute to the expression of the NT/N gene.[37,38]

In certain instances, cells may express a particular mRNA but the protein is not synthesized[39]; therefore, to determine whether NT peptide is produced, colon cancer lines (LoVo, HT29, and HCT116) were extracted and found to have NT peptide by RIA. In addition, we found that NT peptide, in LoVo cells, is released into the culture medium in response to various secretagogues. The compounds 8-bromo-cAMP, a stable cAMP analog, stimulates the release of NT from LoVo cells. Previous studies have indicated that the cAMP pathway is important for the release of NT peptide from endocrine cells.[30,40,41] L-arginine, which stimulates release of other endocrine agents,[42] stimulates release of NT from LoVo in a dose-dependent fashion.

The presence of high-affinity NTR has been demonstrated previously on HT29 cells by binding studies.[43] We next determined whether colon cancers express the mRNA for NTR. As expected, we found expression of NTR mRNA in HT29. In addition, we identified NTR gene expression in the colon cancer line HCT116; however, we were unable to identify NTR expression in LoVo. This may be the result of culture conditions and cell confluency used in this study because Cusack and colleagues[44]

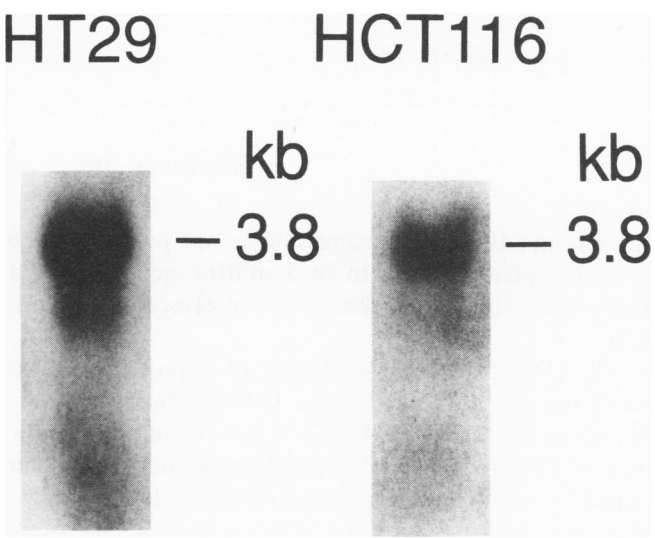


FIG. 4. Expression of the NT receptor mRNA in human colon cancers HT29 and HCT116; 10 μg poly(A)$^+$ RNA was subjected to Northern blot hybridization with the $^{32}$P-labeled rat NT receptor cDNA probe and washed at relatively low stringency. The size of the hybridizing RNA species was derived from the migration of the two large ribosomal RNAs as visualized by ethidium bromide staining.

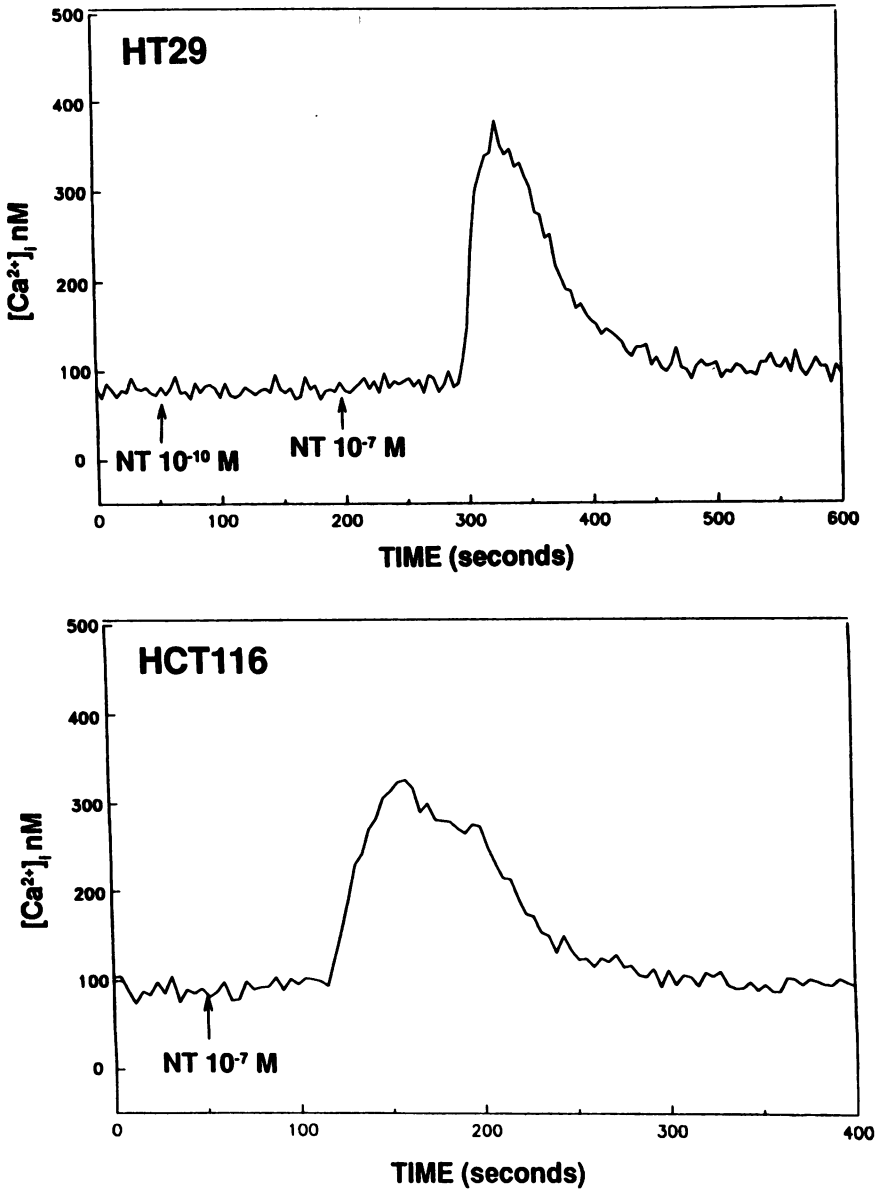


FIG. 5. Mobilization of intracellular calcium ($[Ca^{++}]_i$) in HT29 cells (top) and HCT116 cells (bottom) in response to neurotensin.

have found that NTR expression is not present in the early log phase of growth in a murine neuroblastoma clone, NIE-115, but is present with increased days in culture.

The NTR belongs to the family of G protein-coupled receptors that are linked to the inositol phosphate-$Ca^{++}$ transduction pathway.[29] Calcium plays an important role as a second messenger that regulates numerous cellular activities and triggers a sequence of early cellular changes that culminate eventually in cellular growth.[45] Neurotensin induces a rapid increase in $[Ca^{++}]_i$ and stimulates growth of various small cell lung cancers *in vitro*.[21,22,33,34] Similar to the findings of others,[35,36,44] we have shown that NT stimulates $[Ca^{++}]_i$ in HT29 colon cancer cells. In addition, NT mobilizes $[Ca^{++}]_i$ in HCT116 cells that express the NTR gene. Fetal bovine serum (0.25%) was required, in combination with NT, to cause a small, transient increase of $[Ca^{++}]_i$ in LoVo cells. This requirement of a basal level of FBS in combination with a peptide to effect $[Ca^{++}]_i$ mobilization has been noted for other cell lines.[46]

In conclusion, our study demonstrates the presence of NT/N gene expression, NT peptide content, and functional NTR in various human colon cancers. The overall clinical significance of these findings will require further screening of a large series of primary surgical specimens as well as benign and precancerous lesions. Administration of NT is known to enhance colon carcinogenesis (increased tumor number, size, and incidence of invasion) in rats,[18] stimulate the growth of the colon cancers MC-

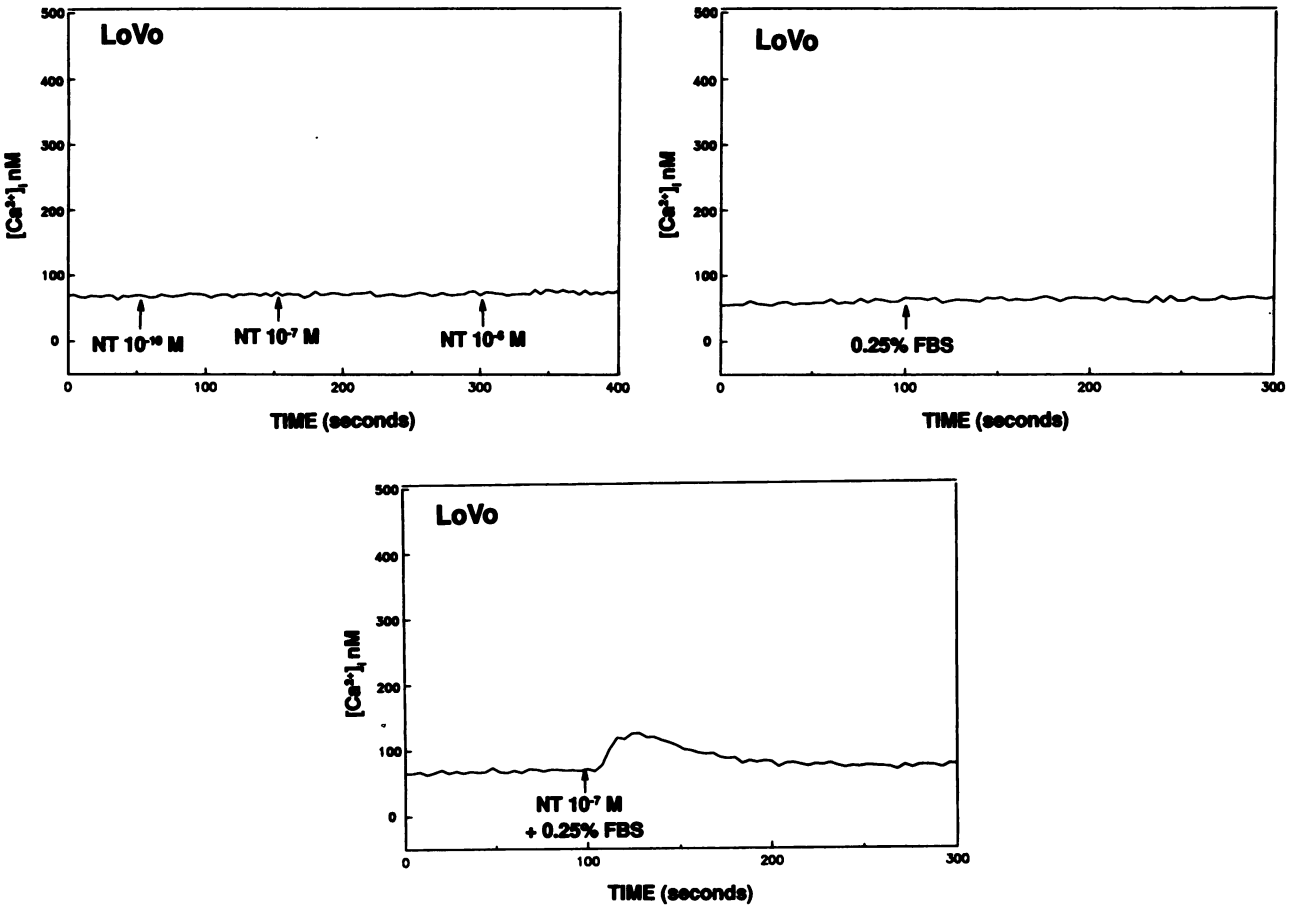


FIG. 6. Ability of NT and fetal calf serum (FCS) either alone (top) or in combination (bottom) to alter intracellular calcium ($[Ca^{++}]_i$) in LoVo cells.

26 and LoVo in nude mice,[19] stimulate $[Ca^{++}]_i$ in colon and lung cancer cell lines,[33-36] and result in the growth of some small cell lung cancers by a presumed autocrine mechanism.[21,22] It is tempting, therefore, to speculate that NT may act as an autocrine factor to stimulate the growth of certain colon cancers; additional studies to evaluate the effect of NT and specific NTR antagonists, which have not yet been developed, on the growth of these colon cancers will be crucial to proving this hypothesis. The identification of patients with colon cancers responsive to NT may have important future implications in the development of therapeutic agents that block the effect of NT.

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## Discussion

Dr. B. Debas (San Francisco, California): Thank you, Dr. Moody. Members and guests: It is a privilege to discuss this paper from the president's unit. It provides me with an opportunity to pay tribute to Jim Thompson and his colleagues.

We heard from Dr. Thompson about the individuals that had the most impact on his life. What he did not talk about was the dozens of individuals whose careers he himself has so powerfully impacted. I am privileged to count myself among the latter.

But no better example of that impact can be provided than by the study so ably presented just now by Dr. Evers. It epitomizes Dr. Thompson's contributions. It is evident that we have an inspired young man in Dr. Evers, who will go on to make his own contributions in the future. The international impact of Dr. Thompson is also evident. One of the co-authors is from Japan, the other from China.

But perhaps most significant is the collaboration with Courtney Townsend. What we are witnessing is the coming to a hot and probing point of Dr. Thompson's enormous interest and expertise in gastrointestinal peptides and Dr. Townsend's interest and experience in oncology.