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Pro-neurotensin/Neuromedin N Expression and Processing in Human Colon Cancer Cell Lines

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The regulatory peptide neurotensin NT has been proposed to exert an autocrine trophic effect on human colon cancers. In the present study, pro-neurotensin/neuromedin N (proNT/NN) expression and processing were investigated in 13 human colon cancer cell lines using a combination of radioimmunoassay and HPLC techniques. All 13 cell lines displayed low to moderate levels of proNT/NN ranging from 10 to 250 fmol/mg protein. However, only 6 (HCT8, LoVo, HT29, C119A, LS174T, and coloDM320) processed the precursor. Three of the latter (HCT8, LS174T, and coloDM320) were analysed in detail with regard to proNT/NN processing pattern and were found to produce NT and large precursor fragments ending with the NT or NN sequence. They had no detectable level of NN. Such a processing pattern resembles that generated by the prohormone convertase PC5. Northern and Western blot analysis of prohormone convertase expression in the 3 cell lines revealed that they were devoid of PC1 and PC2, whereas they all expressed PC5. These data indicate that proNT/NN is a good marker of human colon cancer cell lines while NT is found in only about half of the cell lines. They also suggest that, in addition to NT, several proNT/NN-derived products, possibly generated by PC5, might exert an autocrine positive effect on human colon cancer growth.

Neurotensin (NT) is a regulatory peptide mainly found in the brain, the gut and the adrenals (1-3). Intestinal NT is produced by a discrete population of endocrine cells (N cells) scattered throughout the jejuno-ileal mucosa. The peptide is released in the circulation after food ingestion. Its digestive functions include stimulation of pancreatic and biliary secretions, inhibition of gastric acid secretion and motility, stimulation of colon motility and inhibition of jejuno-ileum motility (reviewed in 4). In addition, NT promotes the growth of gastro-intestinal tissues such as the pancreas, the gastric antrum, and the small and large intestine (5-7). A number of human colon cancer cell lines produce NT and its precursor mRNA (8), and express functional NT receptors (9). Furthermore, NT promotes carcinogenesis in the colon of rats (10) and stimulates the growth of human colon cancer cell lines (8). From these data, it has been hypothesized that NT could act as an autocrine trophic factor in human colon cancer.

Like all neuropeptides, NT is synthesized as part of a larger precursor (11,12), which in addition to NT, contains neuromedin N (NN), a NT-like peptide capable of activating NT receptors. Within the precursor, NT and NN are flanked and separated by Lys-Arg sequences (Fig. 1). These dibasics represent the processing sites recognized by the proprotein convertase (PC) family of enzymes (reviewed in 13,14). Studies by us and others have shown that proNT/NN is differentially processed in the brain, gut and adrenals, giving rise to different combinations of processing products that comprise NT, NN and large peptides ending with the NT (large NT) or NN (large NN) sequence (15,16, see Fig. 1). Recently, we have shown that the prohormone convertases PC1, PC2 and PC5 differentially process proNT/NN with patterns that reproduce those seen in normal tissues (17,18).

Although proNT/NN processing has been examined in human pancreatic and foregut tumor cell lines (19,20), it has not been investigated in human colon cancers. Such studies are important in the above context since proNT/NN can give rise, in addition to NT, to several biologically active peptides that might also activate tumor growth. The present work was designed to investigate the presence and processing of proNT/NN in thirteen human cancer cell lines and to analyse the content in prohormone convertases of those cell lines that processed the precursor.

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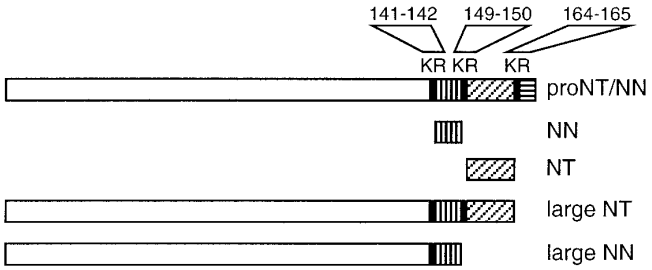

FIG. 1. Schematic representation of proNT/NN and of the peptides detected in tissues that express proNT/NN. Human prepro-NT/NN is 170 amino acid long and starts with a 22 residue signal peptide not represented here. The positions of the Lys-Arg (KR) dibasic sequences are shown. Various combinations of peptides generated by dibasic cleavages are present in tissues that express proNT/NN. Thus, NT and NN are the major products found in the brain, NT and large NN are predominant in the gut, and NT, large NN and large NT are the main products found in the adrenals (15,16).

MATERIALS AND METHODS

Cell culture. The cell lines were cultured, propagated and harvested in the post-stationary growth phase as previously described (9).

Western and Northern blot analysis. Extraction of cells and Western blot analysis were performed as previously described (17). The anti-PC1 antiserum (2B6) and the anti-PC2 antiserum (7BF) used here, both at a 1:1000 dilution, are directed against the amino-terminal portion of their respective antigens. The anti-PC1 antiserum recognizes both the 87 and 66 kDa active forms of the enzyme and the anti-PC2 antiserum detects the 66 kDa mature form of the enzyme (17). BSC40 cells (an African green monkey kidney epithelial cell line) infected with a recombinant vaccinia virus expressing human PC1 (21) and PC12 cells stably transfected with human PC2 (17) were used as positive controls. For Northern blot analysis, total RNA was extracted by the guanidium-phenol-chloroform method and 10 μg were submitted to Northern blot analysis as described (17). PC1, PC2 and PC5 cDNAs were radiolabeled by random priming. PC5-transfected PC12 cells (18) were used as a positive control of PC5 expression.

Radioimmunoassay and citraconylation procedures. The cells were washed with PBS and extracted in ice-cold 0.1 N HCl. The extracts were centrifuged and the supernatants were placed in boiling water for 10 min and then kept frozen until use. The protein content of the extracts was determined using the Bio-Rad protein assay reagent, following the procedure recommended by the manufacturer. The cell extracts were assayed for their immunoreactive NT (iNT) and NN (iNN) contents as previously reported (22,23). The anti-NT and -NN antisera used here recognize the free intact C-terminus and N-terminus of NT and NN, respectively (22). Thus, the NT antiserum does not crossreact with proNT/NN but fully cross-reacts with large NT. The NN antiserum does not recognize precursor fragments, such as large NN, in which the NN sequence is N-terminally extended. In order to detect large NN-containing proNT/NN products, portions of the cell extracts were submitted to Arg-directed tryptic digestion using the citraconylation-trypsin (CT) procedure previously described (22,23). Citraconylated, trypsin-digested samples where then assayed for their iNN content (CTiNN).

Reverse phase HPLC. Cell extracts were fractionated by reverse-phase HPLC as described elsewhere (22,23). The fractions were assayed for their iNT and iNN content. In this system, the retention times for synthetic NT and NN are 41 and 43 min, respectively, and that of large NT is 71 min.

Fractions 60-81, shown in previous studies to contain large precursor forms comprising large NT, large NN and proNT/NN (23), were pooled, subjected to the CT procedure and assayed for their CTiNN content. CTiNN arising from large NN will be in the form of NN while that produced from large NT and proNT/NN will be in the form of NN bearing a C-terminal Lys-Arg extension (NN-KR). To determine the contents of NN and NN-KR, portions of the CTiNN material obtained after the first HPLC were subjected to a second HPLC step as previously reported (23) and the fractions were assayed for their iNN content. In this system, the retention times of NN-KR and NN are 37 and 43 min, respectively.

RESULTS

Cell extracts from thirteen colon cancer cell lines were assayed for their CTiNN, N-terminal iNN and C-terminal iNT contents (Table 1). CTiNN, a measure of the amount of proNT/NN (processed + unprocessed) present at the time of extraction, was detected in all cell lines in amounts ranging from 20 to 240 fmol/mg protein. There was no detectable N-terminal iNN in any of the cell extracts, thus indicating that processing of proNT/NN at the Lys-Arg sequence that flanks the N-terminus of NN did not occur. When assayed with a C-terminally directed NT antiserum, only six of the cell extracts were positive. The ratio of iNT over CTiNN ranged from 9% in HT29 cells to 100% in HCT8 and LS174T, indicating that proNT/NN cleavage at the Lys-Arg sequence that flanks the C-terminus of NT occurred to varying extent in these cell lines.

The three cell lines with the highest levels of iNT, i.e., HCT8, LS174T and coloDM320, were selected for further analysis of proNT/NN-derived products. Cell extracts were submitted to reverse phase HPLC and the fractions were assayed for their iNT and iNN contents. A typical HPLC profile obtained with LS174T cells is shown in Fig. 2. The profiles for the other two cell lines were qualitatively similar. Immunoreactive

TABLE 1

Amounts of iNT, iNN, and CTiNN in Extracts from Human Colon Cancer Cell Lines

Cell line	iNT	iNN (fmol/mg)	CTiNN
SW620	nd[a]	nd	64[b] ± 4
SW48	nd	nd	72 ± 8
SW480	nd	nd	72 ± 6
HCT8	45 ± 5	nd	40 ± 4
HCT116	nd	nd	177 ± 17
LoVo	36 ± 4	nd	82 ± 7
HT29	10 ± 2	nd	118 ± 2
C119A	35 ± 4	nd	66 ± 7
LS174T	93 ± 6	nd	100 ± 10
T84	nd	nd	244 ± 18
Caco2	nd	nd	20 ± 6
colo205	nd	nd	62 ± 4
coloDM320	52 ± 5	nd	238 ± 14

[a] Not detectable.
[b] Mean ± SEM from three independent determinations.

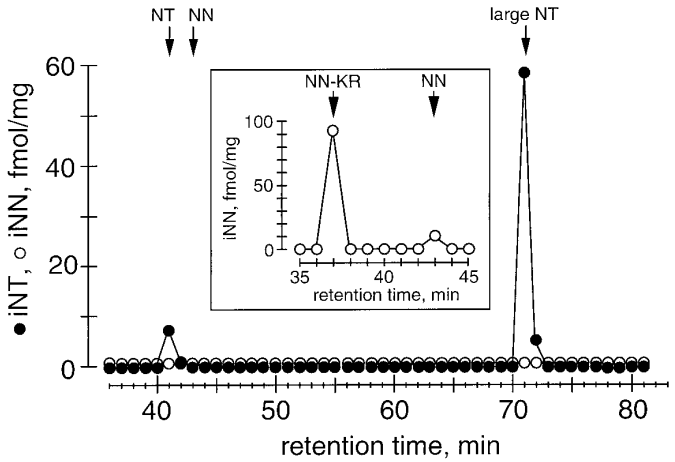

FIG. 2. HPLC separation of proNT/NN derived products. Main figure: an acid extract from LS174T cells (1 to 2 mg protein) was submitted to reverse phase HPLC and aliquot portions of the fractions were assayed for their iNT and iNN content. Arrows indicate the retention times of synthetic NT and NN, and of large NT as determined from previous studies. Inset: fractions from the first HPLC that eluted between 60 and 81 min were pooled, concentrated, subjected to the CT procedure and submitted to a second reverse phase HPLC. The fractions were assayed for their iNN content. Arrows indicate the retention times of synthetic NN and NN-KR. All the data are expressed as fmol of immunoreactive material per mg of protein in the original cell extract.

NT eluted as two peaks with retention times of 41 and 71 min corresponding to those of synthetic NT and large NT, respectively. Fractions 60-81, shown in previous studies to contain large precursor forms comprising large NT, large NN and proNT/NN, were pooled, subjected to the CT procedure and assayed for their CTiNN content. In order to resolve CTiNN in its NN and NN-KR components (see Materials and Methods), the citraconylated material was further fractionated on HPLC and the fractions were assayed for their iNN content. Fig. 2 (inset) shows that approximately 90% and 10% of CTiNN in LS174T cells consisted of NN-KR and NN, respectively. The latter fraction represents large NN while the former is comprised of large NT and proNT/NN. Altogether, these experiments allowed for the quantification of NT, NN, large NT, large NN and proNT/NN in LS174T, HCT8 and coloDM320 cell extracts (Table 2).

The presence of PC1, PC2 and PC5, three prohormone convertases capable of processing proNT/NN, was investigated by Northern blot analysis in the HCT8, LS174T and ColoDM320 cell lines. PC1 and PC2 mRNAs could not be detected in any of the cell lines (not shown). In contrast, Northern blot analysis with a PC5 probe revealed the presence in all three cell lines of moderate levels of a 3.8 kb PC5 mRNA transcript (Fig. 3A). To confirm the lack of PC1 and PC2 expression, immunoblotting experiments were performed using antibodies that crossreact with human PC1 and PC2. Fig. 3B shows that both proteins were undetectable in the three cancer cell lines.

DISCUSSION

The present study demonstrates the presence in thirteen human colon cancer cell lines of proNT/NN. This confirms and extends the data of others (8) who showed that LoVo, HT29 and HCT116 cells expressed proNT/NN mRNA. However, only six of the cell lines examined here produced detectable amounts of iNT. It appears therefore that although proNT/NN expression in human colon cancer cells may be a general phenomenon, the ability of the cells to process proNT/NN and to produce iNT is not.

Detailed analysis of proNT/NN processing in three of the cell lines revealed that they produced essentially large NT, large NN and NT. HCT8 and LS174T cells showed a similar profile of proNT/NN processing (large NT >> large NN = NT) that differed from that found in coloDM320 cells (large NN > large NT >> NT). Despite these differences, all three cell lines are characterized by a high proportion of large NT and by the lack of NN. Such a processing profile differs from that previously reported in brain and gut tissues (15,16). It is however reminiscent of the profile found in the adrenals (16). Recently we showed that PC1, PC2 and PC5 processed proNT/NN with profiles that mimicked those observed in the gut, brain and adrenals, respectively (17,18). It is interesting that the only prohormone convertase that could be detected in the HCT8, LS174T and coloDM320 cell lines is PC5 which yields a pattern of proNT/NN processing comparable to that observed in the cancer cells. It is therefore tempting to speculate that PC5 might play a role in proNT/NN processing in some human colon cancers.

Previous studies have shown that LoVo cells could release iNT in response to cAMP and L-Arginine (8). It may therefore be possible that similar mechanisms operate in HCT8, LS174T and coloDM320 cells to release not only NT but also large NT and large NN. The

TABLE 2

Post-HPLC Determination of NT, NN, Large NT, Large NN, and ProNT/NN Concentrations in Human Colon Cancer Cell Lines

Cell lines	NT	NN	Large NT	Large NN	ProNT/NN[a]
	(fmol/mg)				
HCT8	7.2[b]	nd[c]	34	8.1	9
LS174T	13.2	nd	65	11.3	36
ColoDM320	3.5	nd	45.3	82.4	53

[a] Calculated as the difference between post-HPLC CTiNN and the sum of large NT and large NN.
[b] Mean from two independent experiments.
[c] Not detectable.

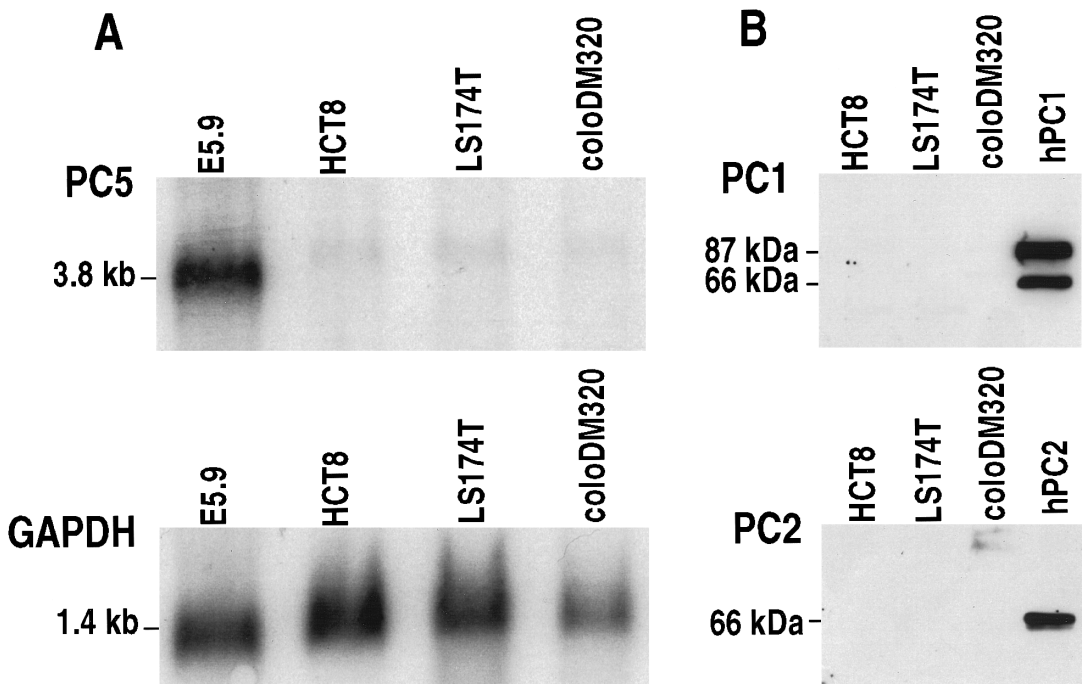

FIG. 3. Northern blot analysis of PC5 and Western blot analysis of PC1 and PC2 in human colon cancer cell lines. (A) Total RNA (10 μg) was analyzed by Northern blotting as described in Materials and Methods. PC5-transfected PC12 cells (clone E5.9) served as a positive control for PC5 expression. Clone E5.9 has been shown to be a strong expressor of PC5 (18), hence the differences in PC5 expression levels observed between clone E5.9 and the cancer cell lines (top). GAPDH mRNA was probed as a control for loading of the gel (bottom). (B) Cell extracts (50 μg of proteins) were analyzed by immunoblotting for the presence of PC1 (top) and PC2 (bottom) as described in Materials and Methods. Human (h) PC1-expressing BSC40 cells (21) and PC2-transfected PC12 cells (17) served as positive controls.

latter product has been shown to activate NT receptors (24). Given the structure of large NT and the pharmacology of NT receptors, it is most likely that this product will also bind to and activate NT receptors. HCT8, LS174T and coloDM320 cells have been shown to express NT receptors (9). Thus, large NN and large NT might both, in addition to NT, participate in the autocrine trophic effect that NT is thought to exert in human colon cancers.

In conclusion, proNT/NN protein expression appears to be a general marker of human colon cancers. ProNT/NN processing, however, occurs in only about 50% of the human colon cancer cell lines examined. This indicates that radioimmunoassays of proNT/NN processing products (such as NT or NN) in human colon cancers may yield negative results even though these cancers express proNT/NN. Furthermore, in those cancer cell lines that process proNT/NN, NT represents a minor product while large molecular forms such as large NN or large NT are more abundant. This points to the necessity of using procedures that can detect these forms in order to assess the potential of human colon cancers to produce proNT/NN-derived autocrine trophic factors. Finally, it may be suggested that agents that could block either NT receptors, like the NT antagonist SR 48692 (25), or proNT/NN processing (PC5 inhibitors yet to be developed) might prove useful in the treatment of human colon cancer.

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