Acetylcholine release by human colon cancer cells mediates autocrine stimulation of cell proliferation

Kunrong Cheng, Roxana Samimi, Guofeng Xie, Jasleen Shant, Cinthia Drachenberg, Mark Wade, Richard J. Davis, George Nomikos, and Jean-Pierre Raufman

Division of Gastroenterology and Hepatology, Veterans Affairs Maryland Health Care System, University of Maryland School of Medicine and Department of Pathology, University of Maryland School of Medicine, Baltimore, Maryland; and Amgen, Neuroscience, Cambridge Research Center, Cambridge, Massachusetts

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Cheng K, Samimi R, Xie G, Shant J, Drachenberg C, Wade M, Davis RJ, Nomikos G, Raufman J-P. Acetylcholine release by human colon cancer cells mediates autocrine stimulation of cell proliferation. Am J Physiol Gastrointest Liver Physiol 295; G591–G597, 2008. First published July 24, 2008; doi:10.1152/ajpgi.00055.2008.—Most colon cancers overexpress M3 muscarinic receptors (M3R), and post-M3R signaling stimulates human colon cancer cell proliferation. Acetylcholine (ACh), a muscarinic receptor ligand traditionally regarded as a neurotransmitter, may be produced by nonneuronal cells. We hypothesized that ACh release by human colon cancer cells results in autocrine stimulation of proliferation. H508 human colon cancer cells, which have robust M3R expression, were used to examine effects of muscarinic receptor antagonists, acetylcholinesterase inhibitors, and choline transport inhibitors on cell proliferation. A nonselective muscarinic receptor antagonist (atropine), a selective M3R antagonist (p-fluorohexahydro-sila-difenidol hydrochloride), and a choline transport inhibitor (hemicholinum-3) all inhibited unstimulated H508 colon cancer cell proliferation by ~40% (P < 0.005). In contrast, two acetylcholinesterase inhibitors (eserine-hemisulfate and bis-9-amino-1,2,3,4-tetrahydroacridine) increased proliferation by 2.5- and 2-fold, respectively (P < 0.005). By using quantitative real-time PCR, expression of choline acetyltransferase (ChAT), a critical enzyme for ACh synthesis, was identified in H508, WiDr, and Caco-2 colon cancer cells. By using high-performance liquid chromatography-electrochemical detection, released ACh was detected in H508 and Caco-2 cell culture media. Immunohistochemistry in surgical specimens revealed weak or no cytoplasmic staining for ChAT in normal colon enterocytes (n = 25) whereas half of colon cancer specimens (n = 24) exhibited moderate to strong staining (P < 0.005). We conclude that ACh is an autocrine growth factor in colon cancer. Mechanisms that regulate epithelial cell production and release of ACh warrant further investigation.

Autocrine signaling; choline acetyltransferase; muscarinic receptors

FOR BOTH MEN AND WOMEN IN the United States colon cancer is a common, frequently lethal disease. Although endoscopic or surgical cancer resection is highly successful in treating early disease, advanced colon cancer responds poorly to surgery, chemotherapy, and radiation, thereby accounting for 30% mortality. A fundamental tenet of cancer biology is that elucidating mechanisms underlying neoplastic cell proliferation will identify therapeutic targets. Hence, identifying colon cancer growth factors and growth factor receptors is a prominent research goal. Nonetheless, although it was recognized more than 15 years ago that muscarinic receptor signaling stimulates colon cancer cell proliferation (9) and that most colon cancers overexpress M3 muscarinic receptors (M3R) (29), muscarinic receptor ligands and receptors have been neglected as potential therapeutic targets.

Postmuscarinic receptor signaling has long been recognized as integral to gastrointestinal physiology. Cholinergic nerve endings are present in colonic mucosa and muscarinic signaling, particularly via M3R, plays a prominent role in mediating intestinal epithelial fluid and electrolyte transport (10, 26, 28, 30). Muscarinic receptor signaling also stimulates colon cancer cell proliferation (6). In human colon cancer cell lines, acetylcholine (ACh)-induced cell proliferation is mediated by M3R-regulated transactivation of epidermal growth factor receptors (EGFR) (4). Our recent finding that genetic ablation of M3R attenuates cell proliferation and neoplasia in a murine colon cancer model further highlights the importance of muscarinic receptor signaling (18).

Over the past 6 years, work from our laboratory demonstrates that luminal bile acids stimulate colon cancer cell proliferation by interacting with M3R and inducing transactivation of EGFR (1–3). The mechanisms that mediate the proliferative actions of bile acids and ACh are indistinguishable (2). By interacting functionally with M3R and causing downstream activation of EGFR and post-EGFR signaling, both ACh and luminal bile acids are growth factors for colon cancer cells that coexpress M3R and EGFR. Whereas the source of bile acids in the colon lumen is evident, it is not apparent that in intestinal mucosa neuronal release of ACh is sufficient to promote proliferation of colon cancer cells. This consideration prompted us to seek alternative intestinal sources of ACh synthesis and release.

ACh is traditionally regarded solely as a neurotransmitter. However, emerging evidence indicates that normal and neoplastic nonneuronal cells also produce and release ACh. Strong evidence for nonneuronal ACh production is reported in human keratinocytes and small cell lung cancer cells (7, 20, 24), indicating in these tissues that ACh may act as an autocrine or paracrine growth factor. Likewise, ACh production by spermatozoa (19), parotid gland epithelium (11), human vascular endothelium (12), and small intestinal mucosa (27) is suggested. However, in these tissues, evidence for ACh production, limited only to demonstrating choline acetyltransferase (ChAT) expression, should be considered inconclusive. Dem-
onstrating ChAT expression alone does not provide sufficient evidence that effective concentrations of ACh have been produced or released.

In the present work, we explored the possibility that nonneuronal production and release of ACh by human colon cancer cells promotes cell proliferation. In our primary test cell line, human H508 cells derived from a well-differentiated cecal adenocarcinoma, we examined the actions of selective and nonselective muscarinic receptor, choline transport, and acetylcholinesterase inhibitors. Nonselective and MsR-selective muscarinic receptor antagonists and a choline transport inhibitor inhibited basal colon cancer cell proliferation. In contrast, increasing concentrations of acetylcholinesterase inhibitors stimulated a progressive increase in cell proliferation. ChAT expression was detected by quantitative real-time RT-PCR (Q-PCR) in H508 and two additional human colon cancer cell lines. Release of ACh into H508 and Caco-2 cell culture media was demonstrated by high-performance liquid chromatography with electrochemical detection (HPLC-ED). These findings indicate that production and release of ACh by colon cancer cells mediates autocrine stimulation of cell proliferation. Moreover, in surgical specimens, upregulated ChAT expression in colon cancer compared with normal colon enterocytes indicates that the molecular machinery regulating nonneuronal ACh synthesis and release may provide novel therapeutic targets.

MATERIALS AND METHODS

Materials. Materials used were purchased as follows: pirenzepine, methoctramine, p-fluorohexahydro-sila-difenidol hydrochloride (pFHHSid), tropicamide, atropine, ACh, hemicholinium-3, bis-9-amino-1,2,3,4-tetrahydroaridine (bis-THA), eserine-hemisulfate, tetracaine, human acetylcholinesterase, and carbamylcholine (carbachol) were from Sigma-Aldrich; mouse anti-ChAT monoclonal antibody, goat anti-mouse IgG, mouse peroxidase/antiperoxidase (PAP), 3,3′-diaminobenzidine (DAB), and osmium tetroxide were from Chemicon; and TRITC-conjugated secondary antibodies were from Sigma-Aldrich. 4,6-Diamidino-2-phenylindole (DAPI) stain for immunofluorescence microscopy was from Vector Laboratories. All other chemicals were obtained from Sigma-Aldrich or Fisher.

Cell lines. H508, HT-29, T84, WiDr, and Caco-2 human colon cancer cells were obtained from the American Type Culture Collection and maintained in culture according to their recommendations. Adherent cultures passed weekly at subconfluence after alignment program and online software (www.genscript.com/sst-bin/app/primer). Q-PCR was performed by use of 7900HT Fast System (ABI) with Power SYBRgreen master mix (ABI), 20 ng primer, and cDNA was synthesized from 50 ng total RNA. PCR conditions included 5 min at 95°C, followed by 45 cycles of 95°C for 1 s, 60°C for 20 s, 72°C for 40 s and a final cycle at 95°C for 15 s, 60°C for 15 s and 95°C for 15 s. PCR data were analyzed using ABI instrument software SDS 2.1. Specificity of amplifications was confirmed by melting-curve analysis. Relative levels of mRNA expression were calculated according to the standard ΔΔCT method. Individual expression values were normalized by comparison with glyceraldehyde-3-phosphate dehydrogenase (gadph). PCR primers from different exons of chat were as follows: forward primer 5′-TTTGTGCTCTC-CACTAGCCA-3′ from exon 17 and reverse primer 5′-ATAACCTT-TGGGACACACG-3′ from exon 18. These exons are common in all known chat isoforms. The length of the Chat-PCR product is 78 bp. PCR primers used for gadph were as follows: forward primer 5′-CCCCATGGTGTCTGAGCG-3′ and reverse primer 5′-CGACAGT-CAGCGCATCTT-3′. The length of the gadph product is 67 bp.

Measurement of colon cancer cell choline and ACh content and release. Choline and ACh concentrations in colon cancer cells and cell culture media were measured by HPLC-ED with a 150 × 3.2 mm column (5 μM particle size). The mobile phase was comprised of 100 mM anhydrous disodium hydrogen phosphate, 0.5 mM tetramethyl ammonium chloride, 2.0 mM 1-octanesulfonic acid sodium salt, 0.05% (vol/vol) reagent MB (ESA) (pH 8.0), adjusted with phosphoric acid) and was delivered by an HPLC pump (ESA) at 0.3 ml/min. Samples were centrifuged at 16,000 rpm for 5 min and supernatants were loaded onto an autosampler (719AL, Alcott) and injected into an HPLC-system that consisted of a solid phase reactor for ACh (ESA; ACH-SPR 70-0640) and an epsilon electrochemical detector (BAS) with a cross-flow, downstream reference-style auxiliary electrode (BAS, MF-1093) and a dual platinum working electrode (BAS, MF-1012; potential = 700 mV, gain = 200 nA). This configuration allowed enzymatic conversion of ACh in the solid-phase reactor followed by electrochemical oxidation of H2O2 that was produced by the enzyme reactions. Areas under the curve were measured by EZ Chrome software (ESA) and compared with single standards for quantification (2 μM for both ACh and choline).

Immunohistochemical analysis. Deidentified surgical sections of human colon tissue from 31 patients, 24 with colon cancer, were obtained from the Department of Pathology at the University of Maryland Medical Center (an exemption for these studies was obtained from the Institutional Review Board on July 16, 2006). Of the 24 adenocarcinoma specimens, 18 were paired with sections of normal colon from the same patient. Following deparaffinization, slices were incubated in 5% goat serum for 2 h and incubated overnight at 4°C with 1:150 dilution of primary antibody (mouse anti-ChAT monoclonal antibody, Chemicon). Slides were incubated with 0.1% TX-100 for an additional 10 min, and blocked for 30 min with PBS/5% serum derived from the same species as the secondary antibody. Cells were incubated overnight at 4°C with the primary antibody (mouse anti-ChAT monoclonal antibody, Chemicon). After incubation, cells were washed in PBS, incubated with secondary TRITC-conjugated antibodies at room temperature for 30 min, and washed. Cell nuclei were visualized with DAPI staining. Slides were analyzed by use of both standard (Nikon Eclipse 80i) and confocal (Zeiss LSM 510) immunofluorescence microscopy.

Cell proliferation. Cell proliferation was determined by the vali-
without addition of primary antibody. Slides were reviewed by a senior pathologist with special expertise in gastrointestinal pathology (C. Drachenberg) masked to sample source. Staining intensity was scored as follows: 0 = negative, 1 = weak, 2 = intermediate, and 3 = strongly positive (17).

Statistical analysis. All figures show data representative of at least three independent experiments. All graphs show means ± SE of at least three independent experiments. Statistical calculations were performed by Student’s unpaired t-test. For immunohistochemistry, scoring for the intensity of ChAT staining was analyzed by the Fisher exact test. Statistical significance is given by the number of asterisks (*P < 0.05; **P < 0.005). P < 0.05 was considered statistically significant.

RESULTS

Actions of muscarinic receptor antagonists and acetylcholinesterase and choline transport inhibitors on cell proliferation. H508 colon cancer cells are derived from a human well-differentiated cecal adenocarcinoma and robustly express M3R but no other muscarinic receptor subtype (5, 6). Consistent with previous observations (2, 6), two cholinergic agonists, ACh and carbachol, reproducibly stimulated H508 colon cancer cell proliferation (Fig. 1A). These actions were blocked by atropine, a nonselective muscarinic receptor antagonist (Fig. 1A). To determine whether constitutive ACh synthesis and release contributes to basal cell proliferation, we examined the actions of maximal concentrations of nonselective and subtype-selective muscarinic receptor antagonists on proliferation of unstimulated H508 cells (Fig. 1B). Atropine, a nonselective antagonist, and pFHHsid, an M3R-selective antagonist, both attenuated unstimulated cell proliferation (Fig. 1B). In contrast, M1R (pirenzepine)-, M2R (methoctramine)-, and M4R (tropicamide)-selective agents did not significantly alter cell proliferation (Fig. 1B). These findings support the hypothesis that release of an endogenous muscarinic agonist stimulates H508 cell proliferation by activating M3R.

Fig. 1. Effects of muscarinic receptor, acetylcholinesterase, and choline transport inhibitors on colon cancer cell proliferation. A: effect of atropine on acetylcholine (ACh)- and carbamylcholine (carbachol)-induced H508 colon cancer cell proliferation. Cells were incubated for 5 days alone or in the presence of ACh (300 μM), carbachol (1 mM), and atropine (1 μM). Absorbance was determined after staining with sulforhodamine-B, as described in MATERIALS AND METHODS (23). B: effects of maximal concentrations of nonselective and subtype-selective muscarinic receptor antagonists on unstimulated H508 cells (Fig. 1B). Atropine, a nonselective antagonist, and pFHHsid, an M3R-selective antagonist, both attenuated unstimulated cell proliferation (Fig. 1B). In contrast, M1R (pirenzepine)-, M2R (methoctramine)-, and M4R (tropicamide)-selective agents did not significantly alter cell proliferation (Fig. 1B). These findings support the hypothesis that release of an endogenous muscarinic agonist stimulates H508 cell proliferation by activating M3R.

Fig. 2. Expression of choline acetyltransferase (chat) mRNA in human colon cancer cell lines. Quantitative real-time RT-PCR (performed as described in MATERIALS AND METHODS) demonstrates abundant expression of chat mRNA in H508, WiDr, and Caco-2 human colon cancer cells, but not in SNU-C4, T84, and HT-29 cells. Results are expressed as means ± SE of at least 3 separate experiments.

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Acetylcholinesterase (AChE) inhibitors attenuate the hydrolysis of ACh. We hypothesized that if ACh release from H508 cells results in autocrine stimulation of cell proliferation, adding AChE inhibitors would stimulate cell proliferation. As anticipated, increasing concentrations of two AChE inhibitors, eserine-hemisulfate and bis-THA, stimulated a robust increase in H508 cell proliferation (Fig. 1C). In contrast, increasing concentrations of tacrine, an agent that is 1,000-fold less potent than bis-THA for inhibiting AChE and is more selective for inhibiting butyrylcholinesterase (15), did not alter cell proliferation (Fig. 1C). Choline is a necessary substrate for ACh synthesis and cellular transport of choline is inhibited by hemicholinium-3. As shown in Fig. 1C, adding increasing concentrations of hemicholinium-3 progressively reduced H508 cell proliferation. Together, the data shown in Fig. 1 support the hypothesis that ACh, produced and released by H508 colon cancer cells, interacts functionally with M₃R, thereby acting as an autocrine growth factor.

Choline acetyltransferase expression in human colon cancer cells. To confirm that H508 cells express ChAT, an enzyme required for ACh formation, we used two experimental approaches: Q-PCR with primers from different exons common to all human isoforms of the chat gene and immunohistochemistry. Expression of chat mRNA was identified in H508, WiDr, and Caco-2 human colon cancer cells (Fig. 2). For comparison, the level of chat expression in H508 cells was set at 1.0 after normalization with gapdh and expression in WiDr and Caco-2 cells was compared with that standard. The chat expression in WiDr and Caco-2 cells, respectively, was ~4- and 65-fold greater than that observed in H508 cells (Fig. 2). In contrast, chat expression was not detected in SNU-C4, T84 and HT-29 human colon cancer cells (Fig. 2). Whereas HT-29 and T84 cell express muscarinic receptors, it appears that SNU-C4 cells express neither M₃R (6) nor ChAT (Fig. 2).

We used immunofluorescence microscopy in colon cancer cells to confirm ChAT expression and to examine its subcellular localization. As shown in Fig. 3A, H508 and Caco-2 cells

![Fig. 3. Expression of choline acetyltransferase (ChAT) in the cytoplasm of human colon cancer cells. A: expression of ChAT in H508 and Caco-2 colon cancer cells is demonstrated by immunofluorescence microscopy. B: confocal microscopy demonstrates cytoplasmic localization of ChAT in representative H508 and Caco-2 cells. Representative brightfield images show typical H508 (a) and Caco-2 cells (e). ChAT immunostaining (red) is evident in the cytoplasm, but not nucleus, of H508 (b–d)and Caco-2 (f–h) cells. Staining with 4,6-diamidino-2-phenylindole (blue) differentiates the nucleus from cytoplasm (c and d for H508 cells; g and h for Caco-2 cells). Scale bars: 100 μm (A), 10 μm (B).](http://ajpgi.physiology.org/)
both demonstrated robust ChAT immunofluorescence. In control cells, incubated in the absence of anti-ChAT antibody, specific binding was not observed (not shown). To examine the cellular distribution of ChAT immunostaining, we used confocal microscopy and nuclear staining. As shown in Fig. 3B, in both H508 and Caco-2 cells (Fig. 3B, b and f), we observed strong cytoplasmic localization of the ChAT signal, confirmed by DAPI counterstaining of the large multilobular nuclei (Fig. 3B, d and h). These observations confirm that expression of chat mRNA (Fig. 2) results in expression of ChAT protein in the cytoplasm of H508 and Caco-2 cells (Fig. 3).

Measurement of acetylcholine and choline in human colon cancer cells. To measure ACh and choline in colon cancer cells, we used a highly sensitive HPLC-ED system (limit of detection for ACh, 5 nM) (21) (representative tracings are shown in Fig. 4, A and B). On the basis of relative chat expression (Fig. 2), we selected three colon cancer cell lines for analysis; H508 and Caco-2 cells which express moderate and high levels of chat, respectively, and HT-29 cells which do not express chat. Applying HPLC-ED, choline, a necessary substrate for ACh production, was detected in both H508 cell extracts and culture medium (11.36 ± 5.35 µM and 5.67 ± 0.74 µM, respectively) (Fig. 4B; Table 1). Choline was also present in the media from HT-29 and Caco-2 human colon cancer cells (Table 1).

Despite adding an AChE inhibitor (0.5 mM eserine), we were unable to detect ACh in H508 cell extracts. Nonetheless, ACh was detected reproducibly in H508 cell culture medium (Fig. 4B; Table 1). We calculated that H508 cells release ~2 nmol ACh/10^6 cells per 24 h. To confirm detection of hydrolyzable ACh, we examined the effects of removing eserine from the culture medium. As shown in Fig. 4C, in the absence of eserine the concentration of ACh plummeted by >95% to nearly undetectable levels. In contrast, as anticipated, the presence of eserine had no effect on the concentration of choline (Fig. 4C). Although abundant choline was present in HT-29 cell extracts and culture medium (Table 1), consistent with the absence of chat expression (Fig. 2), ACh was undetectable (Table 1). Overall, these results confirm that ChAT expression is required for nonneuronal production and release of ACh by colon cancer cells.

ChAT expression in normal colon and colon cancer. To explore further the ability of human colon cancer cells to produce ACh, we used immunohistochemistry to examine colon epithelial ChAT expression in surgical specimens from 31 patients: 25 normal and 24 adenocarcinomas (including 18 normal and cancer specimens from the same patients). ChAT staining was weak or undetectable in normal enterocytes (Fig. 5A; Table 2), but was moderate to strong in 50% of colon cancer specimens (Fig. 5B; Table 2) (P < 0.005; Fisher exact test). In one section, ChAT staining was also detected in metastatic colon cancer cells observed within a lymphatic vessel (Fig. 5C). As observed in human colon cancer cell lines (Fig. 3B), in colon cancer tissue ChAT expression was limited to the cell cytoplasm (Fig. 5D). In resected human colon (25 normal and 24 colon cancers, including 18 paired normal and adenocarcinoma tissues), staining intensity demonstrated upregulated ChAT expression in cancer cells compared with normal colonocytes (P < 0.005) (Fig. 5E). Collectively, these findings indicate that, despite variability in the degree of ChAT expression among colon cancer cell lines (Fig. 2) and colon cancer tissue (Fig. 5, Table 2), ACh production and release is an important manifestation of colon cancer cells.

**DISCUSSION**

In the present communication, in addition to demonstrating expression of ChAT, we used a highly sensitive and specific method (HPLC-ED) to measure directly ACh release from human colon cancer cells. These findings identify a novel

![Table 1. Choline and acetylcholine concentrations detected using high performance liquid chromatography-electrochemical detection in human colon cancer cell culture medium, alone or in the presence of 0.5 mM eserine.](http://ajpgi.physiology.org/)
ACETYLCHOLINE RELEASE BY COLON CANCER CELLS

autocrine growth factor for colon cancer. H508 and Caco-2 human colon cancer cells release in the range of 3 to 7 μM ACh concentrations that can account for basal colon cancer cell proliferation (Fig. 1B) (4). ChAT expression and ACh production are correlated; H508 and Caco-2 cells express chat mRNA (Fig. 2) and Chat protein (Fig. 3) and release ACh (Table 1). HT-29 cells that do not express chat (Fig. 2) do not release detectable ACh (Table 1). Of the six cell lines tested, Caco-2 cells express the most chat mRNA (Fig. 2) and release more ACh than H508 cells (Table 1). ACh release from H508 colon cancer cells was ~25-fold greater than that reported for human small cell lung cancer (SCLC) cells (25) (~2 nmol and ~83 pmol ACh/10⁶ cells per 24 h from H508 and SCLC cells, respectively). As we observed with colon cancer cells, not all SCLC cells release ACh; e.g., HT-29 colon cancer and H417 SCLC cells do not release ACh (Table 1) (25). These observations indicate that Chat expression is related to ACh release from cancer cells.

Likewise, Chat expression varied in surgical specimens. Whereas normal colon showed limited to no Chat staining in enterocytes, half of the colon cancer specimens revealed moderate to strong staining. Only 12 of 24 colon cancer specimens had no Chat staining. The intensity of Chat staining was significantly greater (P < 0.005) in colon cancer compared with normal colonocytes (Table 2). Although the sample size is limited, these findings may have therapeutic implications. For example, expression of estrogen receptors is used to guide breast cancer therapy. In intestinal epithelium, ACh hydrolysis is catalyzed by a highly efficient acetylhydrolase, AChE (EC 3.1.1.7) (22). It is likely that we failed to detect ACh in H508 cell extracts because robust AChE activity rapidly hydrolyzes ACh. Adding eserine, an AChE inhibitor that does not readily penetrate cells (16) increased our ability to detect extracellular (Fig. 4) but not intracellular (Table 1) ACh. Given our ability to measure ACh reproducibly in H508 cell culture medium, we speculate that either the activity of AChE, a membrane-bound enzyme (16), is reduced in cell culture medium compared with cellular levels or that the AChE inhibitor, eserine, did not penetrate cells in sufficient quantity to suppress intracellular AChE activity. At present, we have insufficient information to validate either or both of these plausible explanations.

Decreased AChE activity is reported in colon cancer (13), providing further support for the hypothesis that ACh, whether of neuronal or nonneuronal origin, plays a key role in regulating cell proliferation. As observed following the addition of eserine (Fig. 4C), reduced AChE activity results in concentrations of ACh that stimulate proliferation of human colon cancer cells (Table 1) (4). Hence multiple mechanisms in colon cancer cells can alter extracellular ACh concentration. These include but are not limited to modulation of ACh production (Chat expression and activity), ACh release (intracellular transport and exocytosis), and ACh hydrolysis (AChE expression and activity). In concert with our finding of variable chat expression in colon cancer cells (Fig. 2), variable expression of AChE is another factor that determines the importance of ACh as an autocrine growth factor. In contrast, cellular choline levels are abundant and not likely to limit ACh synthesis. Our findings may help resolve a conundrum posed by the observation that AChE activity is predominantly localized to the apical membrane of Caco-2 cells (16). Muscarinic recep-

Table 2. Expression of Chat in normal enterocytes and colon cancer cells in surgical specimens

<table>
<thead>
<tr>
<th>Chat Staining Score (intensity)</th>
<th>All Cases (N = 49) N (%)</th>
<th>Normal Enteroctyes (N = 25) N (%)</th>
<th>Colon Cancer (N = 24) N (%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (negative)</td>
<td>15 (31)</td>
<td>14 (56)</td>
<td>1 (4)</td>
<td>0.0001</td>
</tr>
<tr>
<td>1 (weak)</td>
<td>22 (45)</td>
<td>11 (44)</td>
<td>11 (46)</td>
<td>NS</td>
</tr>
<tr>
<td>2 (moderate)</td>
<td>10 (20)</td>
<td>0</td>
<td>10 (42)</td>
<td>0.0002</td>
</tr>
<tr>
<td>3 (strong)</td>
<td>2 (4)</td>
<td>0</td>
<td>2 (8)</td>
<td>NS</td>
</tr>
<tr>
<td>Score of 2 or 3</td>
<td>12 (24)</td>
<td>0</td>
<td>12 (50)</td>
<td>0.00003</td>
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</tbody>
</table>

Numbers in parentheses refer to the percentage of cases in each individual column. For example, choline acetyltransferase (Chat) staining was strongly positive in 4% of all tissue, 0% of normal tissue, and 8% of colon cancer. Statistical analysis was performed by using the Fisher exact test (2-tailed) to compare Chat expression in normal enterocytes and colon cancer cells. NS, not significant.
tors are expressed on the basolateral membrane of polarized cells and neuronal ACh should accumulate primarily in this region. Hence previously the functional role of apical AChE was not evident (16). Our finding that colon cancer cells release ACh into the extracellular space indicates that apical AChE may represent an “off” mechanism whereby colon cancer cell ACh is rapidly hydrolyzed after release from the apical pole.

In both health and disease, it is likely that autocrine and paracrine signaling by nonneuronal ACh play an important role in mediating cell function (8, 24). Recent reports that an M3R-selective inhibitor reduced the size of small cell lung cancer xenografts in nude mice (25) and that genetic ablation of M3R reduces murine colon tumor number and size (18) provide additional evidence that muscarinic ligands are key promoters of tumor growth. Our novel findings indicate the likelihood that production and release of ACh by human colon cancer cells stimulates muscarinic receptor-mediated cell proliferation. Further elucidating cellular mechanisms that regulate nonneuronal expression and activation of both ChAT and AChE, and the intracellular transport of ACh are likely to identify novel targets for colon cancer therapy.

REFERENCES