Human Colon Cancer Cell Proliferation Mediated by the M₃ Muscarinic Cholinergic Receptor¹

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ABSTRACT

We have demonstrated previously cell surface receptors for gastrointestinal peptides on 10 human colon cancer cell lines. Because most of the cells studied bind muscarinic cholinergic agonists, we undertook the determination of the cholinergic receptor subtype expressed by human colon cancer cells, as well as the biological function of these receptors, and more specifically, the effect on cell proliferation. We used radiolabeled ligand binding, PCR, calcium mobilization, and cellular proliferation studies. The present study demonstrates a muscarinic cholinergic receptor having two classes of binding site for carbamylcholine. Analysis demonstrated 2499 ± 153 binding sites/cell, of which 75% had a high affinity for carbamylcholine (Kᵦ 55 μM), and 25% had a low affinity (Kᵦ 0.33 μM). N-Methylscopolamine, a receptor antagonist, recognized only one binding site having high affinity (Kᵦ 0.20 μM). The number of muscarinic cholinergic binding sites/cell found on colon cancer cells is 50% of the number of receptors found on guinea pig chief cells in physiological conditions. Specific cholinergic receptor antagonists inhibit binding in the following order of potency: N-methylscopolamine > 4-DAMP > pirenzepine > AF-DX116. This order of potency pharmacologically classifies the receptor as an M₃ subtype. Receptor expression, studied by reverse transcription-PCR, correlates with the binding data. Specifically, cell lines that exhibit binding, abundantly expressed the M₃ receptor subtype, whereas cell lines that do not exhibit binding for muscarinic cholinergic agonists did not abundantly express the M₃ receptor. Agonist activation of the M₃ receptor on these cells resulted in intracellular calcium mobilization. The dose-response curve of calcium mobilization suggests that there are spare receptors on these cells. Signal transduction can be inhibited by receptor antagonists in the same order of potency in which the binding is inhibited. Exogenous agonist added to the cells in culture induces significant cell proliferation. These results demonstrate a muscarinic cholinergic receptor of the M₃ subtype on human colon cancer cells. This receptor induces intracellular calcium mobilization and mediates cell proliferation. The data suggest that there are spare receptors present, and that there may be enhanced intracellular signal activation in response to receptor binding.

INTRODUCTION

Colon cancer is one of the most common solid tumors in the United States, third in both men and women. Great strides have been made in the early detection and prevention of colon cancer, but the treatment options are limited, and the prognosis of patients with advanced disease remains dismal (2, 3). This has prompted investigators to seek new modalities of treatment, as well as an understanding of the mechanism of cancer initiation and progression. A number of genetic abnormalities have been identified and implicated in colon cancer pathogenesis; however, the mechanism of cell growth of colon cancer remains less well understood (4, 5).

Virtually all cancer cells undergo a series of events before transforming into cells capable of growth that is independent of extracellular signals. Among these events are the acquisition of intrinsic growth factor pathways, the establishment of cell autonomy resulting from oncogene abnormalities, the activation of constitutively active signaling pathways, and the loss of cell cycle control resulting from mutations of tumor suppressor genes (6, 7). A requirement for successful tumor progression is the capability of ongoing cell proliferation.

A number of tumors demonstrate abnormalities of cell surface receptors that are of major importance for independent cell growth. One such example is the truncated, constitutively active epidermal growth factor receptor on breast cancers (8). More recently, receptors for a number of gastrointestinal hormones and neurotransmitters have been found on tumors such as breast, prostate, and small cell lung cancer (9–11). Several reports have demonstrated the presence of these receptors on human colon cancer cells and the modulation of cell growth by receptor agonists and antagonists (12–14). To date, however, no reports have studied the mechanism by which these receptors mediate tumor cell growth. It is not known whether augmented cell growth is a result of the receptor-ligand interaction of nonmutated receptors responding to extrinsic or intrinsic ligand or the result of mutated receptors causing constitutively activate signal transduction.

We have shown previously the presence of functional muscarinic cholinergic receptors on 6 of 10 human colon cancer cell lines studied (15). The muscarinic cholinergic receptor is in the super family of G-protein-coupled receptors, which has seven transmembrane domains (16–18). There are five cloned subtypes of the muscarinic cholinergic receptor, which are differentiated by their affinity for specific receptor antagonists,
their tissue localization, and their primary signal-transducing effect (19–24). Two of these receptor subtypes (M1 and M3) have been shown capable of modulating cell growth in certain systems (25). We undertook the experiments described to determine the subtype of muscarinic cholinergic receptor present on human colon cancer cell lines, to evaluate the primary method of signal transduction of the receptor, and to determine whether the receptor subtype was capable of modulating colon cancer cell growth by the normal cell mechanism. These data provide the basis for future studies examining the mechanism of cell growth mediated by these receptors, as well as the possibility of inhibiting tumor growth by interfering with receptor function.

MATERIALS AND METHODS

Cell Culture. Ten previously characterized human colon cancer cell lines were studied (15, 26). Relevant clinical information regarding the primary sites of tumors, sites of the resected tumors from which the cell lines were established, and degree of tumor differentiation have been published previously. Cell lines were grown in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum. Adherent cultures were passaged weekly at subconfluence after trypsinization. Nonadherent cultures were passaged weekly by transfer of floating multicellular aggregates. Cultures were maintained in incubators at 37°C in an atmosphere of 5% CO2 and 95% air. Media and sera were obtained from Grand Island Biological Co. (Grand Island, NY).

Binding of Radiolabeled Ligand. Adherent cells were scraped from the flask with a rubber policeman at least 72 h after last passage using trypsin. Cells were washed twice in a 5-fold volume percentage of medium, spun down at 1000 × g for 5 min, and resuspended in standard incubation buffer consisting of 50 mM Tris buffer, 0.1% bacitracin, 5 mM MgCl2, 130 mM NaCl, 7.7 mM KCl, 1.5 mM EGTA, 4 mg/ml leupeptin, 2 mg/ml chymostatin, and 0.1% BSA at pH 7.4. After washing, the viability of the cells was assessed by trypsin blue exclusion and was >90%, with no evidence of cell lysis. Cells at a concentration of 15 × 10⁶ cells/ml, in a volume of 0.5 ml, were incubated with radioligand at a concentration of 0.6 nM [3H]NMS (70–87 Ci/mmol; New England Nuclear, Boston, MA) for 45 min at 22°C. After incubation with radioligand, cells were sampled, filtered using glass microfiber filters (GF/C; Whatman International Ltd., Maidstone, England), and then counted in a liquid scintillation spectrometer (Packard Instrument Corp., Sterling, VA).

Nonsaturable binding was the amount of radioactivity associated with the cells when the incubation contained radioligand plus the unlabeled peptide at concentrations of 1–10 nM. Nonsaturable binding of <15% was exhibited in all cases. Values shown for saturable binding are those measuring binding with radioligand alone (total binding) minus the nonsaturable binding. All values are for saturable binding unless stated otherwise. Dissociation constants were determined by the nonlinear, least-squares curve-fitting program (LIGAND; Ref. 27). The inhibitory constant values for antagonist binding and their relationship to the IC50 were calculated by the method of Cheng and Prusoff (28). Scatchard plot was determined by the curve-fitting program (LIGAND; Ref. 27).

RT-PCR Receptor Expression. RNA was extracted using a modified acid guanidinium-isothiocyanate technique (29). RNA concentration was determined by 260/280 UV absorbency, and RNA quality was checked by agarose gel electrophoresis. Qualitative differences in expression for the five subtypes of muscarinic cholinergic receptor were determined by comparing PCR gene products to an internal standard (β-actin). A typical reverse transcription reaction consisted of: 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 2.5 mM random primers (Pharmacia, Piscataway, NJ), 200 mM of each deoxyribonucleotide triphosphate, 100 units of SuperScript II RNase H-Reverse Transcriptase (Life Technologies, Inc., Bethesda, MD), and 1000 ng of RNA. The reaction conditions were 10 min at room temperature, 30 min at 42°C, 5 min at 99°C, followed by 5 min at 5°C. After the reverse transcriptase reaction, PCR was initiated by the addition of 5 μl of reverse transcriptase product to PCR reagent mix. The final concentration of PCR reagents was: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.5 mM of each primer, and 2.5 units Taq DNA Polymerase (Life Technologies). The reaction conditions were 94°C for 30 s, 65°C for 30 min, and 72°C for 30 s, for 30 cycles. After PCR amplification, 8.5 μl of each reaction were resolved on a 2% Metaphor agarose gel (FMC, Rockland, ME) containing 0.5 mg/ml of ethidium bromide.

Primers were synthesized by the DNA core facility at the Fox Chase Cancer Center. Primer sequences were derived from GenBank sequences for each receptor subtype and are listed in Table 1. Parental CHO3 and transfectants expressing the M1–M4 muscarinic cholinergic receptor subtypes were kindly provided by Mark Brann (Acadia Pharmaceuticals, San Diego, CA).

Intracellular Calcium Mobilization. Intracellular calcium concentrations were measured using the fluorescence in-

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<thead>
<tr>
<th>Table 1</th>
<th>Oligonucleotide primers used for RT-PCR receptor expression</th>
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<tr>
<td>mACH receptor isoform</td>
<td>Primer sequence</td>
</tr>
<tr>
<td>M1 (M35128)b</td>
<td>5′-gagcaagctcgtacctgcc-3′</td>
</tr>
<tr>
<td>M2 (16104)b</td>
<td>5′-catccgtccctcctcctc-3′</td>
</tr>
<tr>
<td>M3 (15266)b</td>
<td>5′-gagacgacagagagctacctc-3′</td>
</tr>
<tr>
<td>M4 (X15265)b</td>
<td>5′-actgtcctgagccacgctcctc-3′</td>
</tr>
<tr>
<td>M4 (M80333)b</td>
<td>5′-cctggtgacgacaggtgagc-3′</td>
</tr>
</tbody>
</table>

aThe muscarinic cholinergic receptor subtype, sense and antisense primer sequences, fragment length, and GenBank accession number for each receptor isoform is shown.

bGenBank accession number.
Fig. 1 Ability of carbamylcholine to inhibit binding of [3H]NMS to colon cell line NCI-H508. Cells were incubated at 22°C for 46 min with 0.6 nM [3H]NMS plus the indicated concentration of ligand. Binding is expressed as a percentage of [3H]NMS that was saturably bound in the absence of ligand. Points, means from at least four experiments; bars, SE. Inset, Scatchard analysis derived from the data points using the nonlinear, least-squares curve fitting program (LIGAND).

Fig. 2 Ability of the muscarinic cholinergic antagonists to inhibit binding of [3H]NMS to colon cell line NCI-H508. Cells were incubated at 22°C for 46 min with 0.6 nM [3H]NMS plus the indicated concentrations of ligands. Binding is expressed as a percentage of [3H]NMS that was saturably bound in the absence of ligand. Points, means from at least four experiments; bars, SE.

RESULTS
Pharmacological Characterization of Receptor Subtype. Radiolabeled ligand-binding studies demonstrated that 6 of 10 human colon cancer cell lines exhibited a significant amount of binding of [3H]NMS with a mean value of 1332 fmoles/μg DNA (range, 314-3443 fmoles/μg DNA; Ref. 15). There were no phenotypic differences between cells exhibiting receptor compared with those that did not exhibit receptor binding. To further examine the pharmacological properties of the muscarinic cholinergic receptor on these cells, we used cell line NCI-H508 as a model for cells exhibiting receptor binding. This cell line was derived from a moderately differentiated adenocarcinoma of the cecum that had metastasized to the abdominal wall.

The muscarinic cholinergic agonist, carbamylcholine, and the muscarinic cholinergic antagonist NMS were tested for their abilities to inhibit binding of [3H]NMS. With carbamylcholine, detectable inhibition occurred at a concentration of 10 μM, half-maximal inhibition at 300 μM, and complete inhibition at 30 μM (Fig. 1). Computer analysis of the broad dose-inhibition curve of [3H]NMS binding by carbamylcholine was best fit with a model having two classes of binding sites (Fig. 1). Computer analysis demonstrated 2499 ± 153 binding sites/cell (mean ± SE, n = 6), of which 75% had a high affinity for carbamylcholine (Kd 55 ± 7 μM), and 25% had a low affinity (Kd 0.33 ± 0.03 μM). With NMS, detectable inhibition of radioligand binding occurred at a concentration of 100 pM, half-maximal inhibition at 1 nM, and complete inhibition at 1 μM (Fig. 2). Computer analysis of the dose-inhibition curve of [3H]NMS by NMS was best fit by a model having a single class of binding sites, demonstrating that the antagonist could not distinguish between the muscarinic cholinergic receptors having high and low affinities for the agonist. The analysis demonstrated 2499 ± 153 binding sites/cell (mean ± SE, n = 6) having a Kd of 0.20 ± 0.04 μM.
Table 2  Ability of various muscarinic cholinergic antagonists to inhibit binding of [H]NMS to human colon cancer cell line NCI-H508

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>[H]NMS binding, K (nM)</th>
</tr>
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<tr>
<td>NMS</td>
<td>0.5 ± 0.07</td>
</tr>
<tr>
<td>4-DAMP</td>
<td>14.0 ± 0.4</td>
</tr>
<tr>
<td>Pirenzipine</td>
<td>3,000 ± 487</td>
</tr>
<tr>
<td>AF-DX 116</td>
<td>20,000 ± 843</td>
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To characterize the subtype of muscarinic cholinergic receptor demonstrated, various receptor antagonists were tested for their abilities to inhibit binding of [H]NMS. Pharmacological studies have demonstrated that distinct receptor subtypes possess high affinity for specific receptor antagonists (23). The M₁ (cerebral type) receptor has a high affinity for pirenzipine, the M₂ (cardiac type) has a high affinity for AF-DX116, and the M₃ (glandular type) has a high affinity for 4-DAMP. In human colon cancer cells, the antagonists had inhibitory constants as follows: NMS = 0.5 nM > 4-DAMP = 14 nM >> pirenzipine = 3 μM > AF-DX116 = 20 μM (Table 2). This pharmacologically classifies the muscarinic cholinergic receptor found on human colon cancer cells as an M₁ (glandular) type of receptor.

**Molecular Classification of Receptor Subtype.** Radio-labeled ligand binding studies allow for the differentiation of three classes of muscarinic cholinergic receptor subtypes based on their affinity for receptor antagonists (32). The genes for five subtypes of muscarinic cholinergic receptor have been cloned (32). The widespread distribution of these receptor subtypes and their diverse binding properties suggest that radiolabeled ligand binding studies assess the properties of heterogeneous combinations of receptor subtype (23). To determine whether human colon cancer cell lines express the glandular receptor subtype or a combination of receptor subtypes, we studied the cells for transcript expression of each of the five receptor subtypes.

Using the known sequences of the five muscarinic cholinergic receptor subtypes, designated M₁–M₅ and m₅, we designed primer sets to amplify a nonhomologous region of each receptor subtype. Parental CHO cells were used as a negative control, and CHO cells expressing the M₁–M₅ and m₅ receptor subtypes were used as positive controls. Each oligonucleotide primer set described amplified only the RNA from the transfected cell line expressing the receptor subtype for which it was designed. The parental cell line RNA was not amplified by any of the primer sets. The colon cancer cells did not express transcript for regions of the M₁, M₅, or m₅ receptor subtypes. Transcript for the M₅ receptor subtype was expressed in all cell lines but at very low density, and there was no ligand binding to this receptor. Transcript for the M₁ receptor subtype was abundantly expressed by cell lines H-498, H-508, H-548, H-716, H-768, and SNU-C1 but not for cell lines H-630, H-747, SNU-C4, or SNU-C5 (Fig. 3 and Table 3). There was good correlation between M₁ receptor transcript expression and the radiolabeled ligand binding studies, demonstrating that these receptors are functional when present on the cell membrane. To determine whether M₃ receptors were present on normal colon tissue, we studied resected colon tissue from patients undergoing surgery. Matched pairs of colon cancer and normal colon tissue were obtained from eight patients. RNA was isolated as described. All normal and cancer tissues expressed the M₃ receptor. In six of these patients, the quantity of receptor expressed in cancer tissue was 3–10-fold greater than in adjacent normal tissue. In the remaining two patients, the quantity of receptor expressed was similar. This demonstrates that the M₃ receptor is found on normal and malignant colon tissue but is frequently overexpressed in cancer tissue.

**Receptor-Ligand-mediated Cytosolic Calcium Mobilization.** The muscarinic cholinergic receptors belong to the class of seven transmembrane domain, G-protein-mediated cell surface receptors (16). When activated by receptor-ligand interaction, G-proteins mediate diverse cellular events, including the inhibition of adenylyl cyclase activity, the stimulation of phosphoinositide breakdown, the regulation of inward potassium current, and cytosolic calcium mobilization (33–37). The primary signal-transducing effect of the M₂ and M₄ receptor subtypes is adenylyl cyclase inhibition, whereas the primary effect of the M₁, M₃, and m₅ receptor subtypes is phosphoinositide turnover and calcium mobilization (24). To determine whether occupation of the M₃ receptor expressed on human colon cancer cells results in the expected biological activity, we studied the ability of receptor agonists and antagonists to alter intracellular calcium in cell line H-508.

Carbamylcholine caused a rapid transient increase in [Ca²⁺], (Fig. 4). The increase was detectable at a concentration of 0.1 μM, with an 18 ± 4% (n = 5) maximum increase above baseline (Fig. 5). Maximal [Ca²⁺], mobilization was detected with 1 mM carbamylcholine, and half-maximal stimulation was detected with 20 μM carbamylcholine (Fig. 5).

To establish the effect of receptor inhibition on the biological activity mediated through the cholinergic receptor on cell line H-508, we studied the ability of specific receptor antagonists to inhibit cytosolic calcium mobilization. All of the antagonists used were capable of completely inhibiting the calcium mobilization induced by 1 mM carbamylcholine (Fig. 4). One hundred % inhibition was accomplished by the addition of 1 μM 4-DAMP, which was 10-fold more potent than pirenzipine (requiring 10 μM), and 100-fold more potent than AF-DX116 (requiring 0.1 mM; Fig. 4).

The results of the calcium mobilization experiments demonstrate that the muscarinic cholinergic receptor expressed on human colon cancer cells is biologically active. Because calcium mobilization is the primary signal-transducing effect of this receptor and the most potent antagonist is 4-DAMP, this confirms that the receptor subtype is M₃ (glandular).

**Receptor-mediated Cell Proliferation.** It has been shown recently that G-protein-coupled receptors are capable of modulating cell growth (6). Muscarinic cholinergic receptors are among the G-protein-coupled receptors which when activated, can induce cell proliferation and transformation. This observation is cell type dependent and receptor subtype specific. To date, only the M₁ and M₄ receptor subtypes have demonstrated growth modulation (25). Because the muscarinic cholinergic receptor found on human colon cancer cells is of the M₃ subtype, we used a proliferation assay to evaluate its growth-
modulating potential in this system, using cell line NCI-H508 as a model for cells exhibiting the M₃ receptor.

A standard curve correlating absorbance to cell number had a linear regression coefficient of 0.95. Cell cycle synchronization was accomplished by plating cells in medium containing no serum. The cells were incubated with increasing concentrations of carbamylcholine, a receptor agonist. After 6 days of incubation in H508 cells, a detectable 25±6% (mean±SE, n=5) augmentation of cell proliferation above control was observed at a concentration of 0.1 mM carbamylcholine (Fig. 6). Microscope visualization and examination of plated cells before each SRB assay confirmed an increase in the number and the density of cells compared with control. A dose-response relationship was maintained for all concentrations of carbamylcholine used. The proliferative effect was statistically significant at concentrations of carbamylcholine above 30 mM. The maximum proliferative effect observed was a 133±15% (mean±SE, n=5) augmentation of cell proliferation above control at a concentration of 1 mM carbamylcholine (Fig. 6). Simultaneous incubation of cells with 0.3 mM of carbamylcholine and increasing concentrations of NMS demonstrated a dose-response inhibitory effect of NMS on carbamylcholine-induced cell growth. Cells not exhibiting the M₃ receptor did not demonstrate augmented cell proliferation when incubated with receptor agonist.

These proliferation experiments demonstrate that the M₃ (glandular) subtype of muscarinic cholinergic receptor found on human colon cancer cells is capable of mediating a growth response.

### DISCUSSION

By definition, cancer cells are transformed and are presumed not to require external stimuli for proliferation or perpetuation. Many growth factor receptors have been implicated in cancer cell growth, but these are usually receptor mutations that result in a constitutively active state (6). To date, these receptors have been of the intrinsic tyrosine kinase-mediated superfamily of receptors, such as the epidermal growth factor receptor. The gastrointestinal tract is the largest endocrine organ in the human body and is constantly affected by peptide hormones and neurotransmitters acting on receptors belonging to the seven transmembrane domain, G-protein-mediated superfamily of receptors. Many of these peptide hormones and neurotransmitters have growth effects on normal and neoplastic tissue; however, few studies have investigated the role of the muscarinic cholinergic receptors on gastrointestinal cell growth. No studies have examined the effect of muscarinic cholinergic receptor activation on growth of human colon cancers.

We have shown previously that a large number of human colon cancer cell lines possess muscarinic cholinergic receptors, a G-protein-mediated type of receptor (15). The receptor ligand...
is a common neurotransmitter of the gastrointestinal tract. The present studies were undertaken to classify the subtype of muscarinic cholinergic receptor found on human colon cancer cells and to investigate the functional effect of this receptor on colon cancer cell biology. The present results demonstrate a ligand-mediated growth modulation of colon cancer cells. This effect is mediated by the M3 (glandular) subtype of muscarinic cholinergic receptor, using intracellular calcium as the transducing signal.

In 1914, cholinergic receptors were classified as either muscarinic or nicotinic, based on the mediated effects of acetylcholine (38). Pharmacological studies using muscarinic cholinergic receptor antagonists suggested the presence of three distinct receptor subtypes (32). Subsequently, five subtypes of muscarinic cholinergic receptor were cloned and sequenced (32). This had the effect of enabling receptor subtype categorization by sequence, signal transduction effect, and ligand affinity. Although precise receptor subtype classification is now possible, several nomenclatures exist, and proper receptor identification requires study of receptor pharmacology, expression, and function. The most current classification for the muscarinic cholinergic receptors is the 1998 TiPs nomenclature, which is used in this study (39).

In the present study, radiolabeled ligand binding demonstrates a receptor for muscarinic cholinergic agents on most human colon cancer cells. Computer analysis of the binding revealed two classes of binding sites that have different affinities for the agonist, carbamylcholine, but the same high affinity for the antagonist, NMS.

Pharmacological studies have used a panel of receptor antagonists to differentiate the muscarinic cholinergic receptor subtypes. The M1 receptor subtype, typically localized to the cerebral cortex, exhibits a high affinity for pirenzipine and a low affinity for AF-DX116 (23). The M2 receptor subtype, typically localized to cardiac tissue, exhibits a high affinity for AF-DX116 and a low affinity for pirenzipine (23). The third receptor subtype classified by pharmacological studies is the M3 receptor, typically localized to glandular epithelium (23). This receptor has also been labeled the m3, M4, and the M2 receptor (24). It exhibits a high affinity for 4-DAMP and a low affinity for pirenzipine and AF-DX116. The receptor on the human colon cancer cell lines studied has a high affinity for 4-DAMP and a very low affinity for AF-DX116 (39), resulting in difficult pharmacological differentiation from the M3 receptor.

The results of the RT-PCR experiments were in concordance with the binding data demonstrating an M3 receptor. The cell lines exhibiting binding of carbamylcholine abundantly expressed the M3 receptor subtype mRNA, whereas the cell lines not exhibiting binding of carbamylcholine did not. For these functional receptors, the biochemical binding is attributable to M3 receptor expression. In addition, study of human colon...
tissue reveals an overexpression of the M₃ receptor on cancer cells compared with normal colon tissue.

The M₃ receptor is a G-protein-mediated receptor that induces intracellular calcium mobilization when activated (24). The present data demonstrate a dose-response correlation between agonist binding and calcium mobilization for the receptor studied. Receptor antagonists were capable of eliminating the signal-transducing effect of agonist in the following order of potency: 4-DAMP > pirenzepine > AF-DX116. This confirms the pharmacological classification of the receptor as an M₃ subtype.

Intracellular calcium mobilization was detectable with 0.1 μM agonist, half-maximal stimulation was seen with 10 μM agonist, and maximal stimulation was seen with 1 mM agonist. Using carbachol to inhibit radiolabeled ligand binding, detectable inhibition occurred at 10 μM, half-maximal inhibition was at 300 μM, and complete inhibition was at 30 μM. Carbachol was 30–100-fold more potent at stimulating intracellular calcium mobilization than interacting with the high-affinity site of the receptor. This phenomenon suggests the presence of spare receptors for muscarinic cholinergic ligands on human colon cancer cells. An observation of this type implies a physiologically relevant effect of receptor binding on cell function (40).

Muscarinic cholinergic receptors are capable of inducing cell proliferation. This capability is receptor subtype specific and has been observed for the M₁ and M₃ subtypes (25). The results of the colorimetric growth assays using human colon cancer cells demonstrate a significant effect on cell proliferation by receptor agonist. The dose-response effect demonstrated for the agonist on cell proliferation is very similar to that seen for intracellular calcium mobilization. An increase in cell growth is detectable with 0.1 μM carbachol, and a maximal growth effect is observed with 1 mM carbachol. This provides evidence that the M₃ receptor found on human colon cancer cells is functional and capable of mediating cell proliferation using intracellular calcium as the signal-transducing effect.

It has been suggested previously that a high cholinergic receptor density is needed for agonists to mediate cell proliferation (25). It has been further postulated that receptors efficiently couple to growth-regulatory pathways only when they are overexpressed. We have determined that there are 2499 ± 153 muscarinic cholinergic binding sites/cell on human colon cancer cell lines. A highly enriched preparation of chief cells prepared from guinea pig stomach demonstrates 5920 ± 953 muscarinic cholinergic binding sites/cell (40). This would suggest that the M₃ receptor found on human colon cancer cells may be extremely efficient in terms of its growth-regulatory pathway.

In summary, the present data demonstrate an M₃ subtype of muscarinic cholinergic receptor on human colon cancer cells. The mechanism of signal transduction is intracellular calcium mobilization, which has a positive proliferative effect. The results provide a basis for further study including: investigation of enhanced receptor activation and signal transduction by the M₃ receptor; possible interventions to inhibit growth; and demonstration of the effect of receptor activation on nuclear genetics and tumor progression.

REFERENCES


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