Muscarinic receptors and ligands in cancer

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Shah N, Khurana S, Cheng K, Raufman JP. Muscarinic receptors and ligands in cancer. Am J Physiol Cell Physiol 296: C221–C232, 2009. First published November 26, 2008; doi:10.1152/ajpcell.00514.2008.—Emerging evidence indicates that muscarinic receptors and ligands play key roles in regulating cellular proliferation and cancer progression. Both neuronal and nonneuronal acetylcholine production results in neurocrine, paracrine, and autocrine promotion of cell proliferation, apoptosis, migration, and other features critical for cancer cell survival and spread. The present review comprises a focused critical analysis of evidence supporting the role of muscarinic receptors and ligands in cancer. Criteria are proposed to validate the biological importance of muscarinic receptor expression, activation, and postreceptor signaling. Likewise, criteria are proposed to validate the role of nonneuronal acetylcholine production in cancer. Dissecting cellular mechanisms necessary for muscarinic receptor activation as well as those needed for acetylcholine production and release will identify multiple novel targets for cancer therapy.

tumor genesis; nonneuronal; cholinergic signaling; acetylcholine; bile acids

EFFICIENT INTERCELLULAR and intracellular signaling is critical for successful proliferation and survival of neoplastic cells. Depending on cancer cell type, a host of important receptors, ligands, signaling pathways, and molecules have been identified. From a clinical perspective, elucidating these signaling mechanisms is key to developing targeted, effective, and safe cancer therapeutics. In normal mammalian physiology, the critical importance of acetylcholine signaling via muscarinic receptors has long been recognized in neuronal tissue. However, the role of muscarinic signaling in neoplasia has received relatively scant attention. Recent observations, reviewed critically herein, prompted the present comprehensive review of the role of muscarinic receptors and ligands in cancer and an appraisal of the potential therapeutic implications of these experimental findings.

Acetylcholine was first discovered by Sir Henry Dale in 1914, and its function as a neurotransmitter was confirmed in 1926 by Otto Loewi (124). In both the central and peripheral nervous systems, acetylcholine plays a pivotal role in neuronal signaling (82, 83, 129). On the basis of the binding affinity of two naturally occurring substances, muscarine and nicotine, cholinergic receptors are generally classified in two broad categories, muscarinic or nicotinic. The discovery of more selective agonists and inverse agonists, along with the advent of gene cloning, led to the identification of five muscarinic receptor subtypes (designated M1R–M5R). In the present review, we focus almost exclusively on the role of muscarinic receptor signaling in cancer. Readers interested in nicotinic signaling may refer to recent excellent reviews (35, 48, 55).

It is not a novel concept that, in addition to neuronal production and release at nerve synapses, acetylcholine can be produced and released by nonneuronal cells in sufficient quantity to modulate cell function (9, 129). Nonetheless, over the past few years, a surge of information pertains to nonneuronal production and release of acetylcholine by neoplastic cells. Moreover, novel observations support the importance of muscarinic receptor expression and activation in cancer. We examined critically the purported role of muscarinic receptor signaling in the progression of neoplasia and, on the basis of the resulting analysis, propose novel criteria to validate the importance of both muscarinic signaling and nonneuronal acetylcholine production in cancer.

MUSCARINIC RECEPTORS

In 1869, Schmiedeberg and Kopp showed that increasing concentrations of extracts of the mushroom

Amanita muscaria

progressively slowed and arrested beating of frog hearts (17). It was discovered later that these effects were mediated by muscarinic receptors, members of a large receptor superfamily that activate guanosine 5’-phosphate (G) proteins (G protein-coupled receptors) (17). G proteins modulate the activity of adenyl cyclase, ion channels, and phosphatidylinositol lipid turnover, thereby regulating a broad repertoire of biological responses (51, 85).

Structure and Subtypes of Muscarinic Receptors

As illustrated in Fig. 1, muscarinic receptors are composed of seven transmembrane helical domains connected by three extracellular and three intracellular loops. Initially, on the basis of pharmacological selective affinity to the muscarinic agonist pirenzepine, two subtypes of muscarinic receptors, M1R and M2R, were described (61). In 1987, using molecular cloning techniques, Bonner et al. (14) described five muscarinic recep-
Muscarinic receptors are expressed in various organ systems, and, in fact, expression of these critical signaling molecules may be ubiquitous. All five muscarinic receptor subtypes are expressed in brain (1). Likewise, all five muscarinic receptor subtypes are expressed in the eye, but M3R predominate (49). M2R and, to a lesser extent, M3R regulate ciliary muscle contraction, whereas M2R regulate pupillary constriction (13, 20, 32, 87). M1R, M3R, and M4R are expressed in the heart (126); M3R activation results in bradycardia and reduced inotropy, whereas M1R activation results in tachycardia (1, 33, 37, 67, 118). Muscarinic receptor activation produces vasodilation in cerebral, coronary, and systemic vasculature (73, 135). While M1R activation produces relaxation of systemic vascular tone (73), M3R activation relaxes cerebral vascular tone (135). In various organs, including the gastrointestinal tract (30, 39, 52, 86), lung bronchioles (45), urinary bladder (29, 62, 125), and uterus (74), smooth muscle contraction is mediated by a mixture of M1 and M3 receptors. Whereas activation of M2R appears to be the primary regulator of smooth muscle contraction, activation of M4R potentiates this action (74).

In gastrointestinal tissue, the primary muscarinic receptor subtypes are M1R, M3R, and M4R. In salivary glands, M3R regulate flow and volume of secretion, whereas M1R regulate release of high-viscosity saliva (50, 65). M3R regulate acid secretion from gastric parietal cells (38, 113), and a mixture of M1R and M3R regulate pepsinogen secretion from chief cells (103, 119, 134). M1R also play a dominant role in regulating fluid and electrolyte secretion from enterocytes (18, 66, 120, 138, 139).

**MUSCARINIC RECEPTOR LIGANDS AND SIGNALING**

In humans, the only naturally occurring endogenous muscarinic receptor agonists are acetylcholine (ACh) and conjugated secondary bile acids. ACh production and release by both neuronal and nonneuronal cells is reported; these will be described separately. Evidence for the role of bile acids in muscarinic receptor signaling is also discussed in this section.

**Neuronal Signaling by ACh**

As illustrated in the overview of neuronal ACh production and release shown in Fig. 2, key components of neuronal ACh production and degradation include 1) plasma membrane choline transporters; 2) cytoplasmic choline acetyltransferase (ChAT), an enzyme that catalyzes formation of ACh from choline and acetyl coenzyme A (acyetyl CoA) substrates; 3) cytoplasmic ACh transporters; and 4) cholinesterases that catalyze rapid ACh hydrolysis. Choline is actively transported into neurons by means of the choline transporter ChT-1. In a reaction catalyzed by ChAT, intracellular choline combines with acetate (derived from acetyl CoA), to form ACh and CoA. Assisted by vesicular transporters VACH and ChT-1, newly formed ACh is stored in membrane vesicles that are transported along the axon. Following neuronal stimulation, these
vesicles fuse with the plasma membrane, thereby releasing ACh into the synaptic cleft where it can interact with both muscarinic and nicotinic ACh receptors. Tissue and released acetylhydrolases [acetylcholinesterase (AChE), EC 3.1.1.7, and butyrylcholinesterase (BChE), EC 3.1.1.8] rapidly hydrolyze free ACh to form choline, acetate, and water. For ACh hydrolysis, AChE has 1.5 to 60 times greater activity than BChE, and, in humans, AChE comprises >95% of cholinesterase activity (7). Nonetheless, in rodents, inhibition of BChE results in a large increase in brain ACh (34). Hence, the enzyme primarily responsible for ACh hydrolysis may vary depending on the organ and species examined.

Nonneuronal Signaling by ACh

ACh released from nerve endings is rapidly hydrolyzed by cholinesterases, thereby limiting its actions to immediately neighboring cells. Hence, ACh production by nonneuronal cells can play a key role in regulating actions of cells or tissues that are not innervated by cholinergic neurons (e.g., breast and bronchial epithelial cells). To describe the production of ACh by nonneuronal tissues, independent of neuronal input, the term “nonneuronal ACh” was coined. In contrast to release of neuronal ACh at synaptic clefts, nonneuronal ACh signaling may not require expression of choline or vesicular transporters, and released ACh acts in an autocrine or paracrine mode, thereby regulating the function of ACh-releasing or neighboring cells, respectively (Fig. 3). Although expression of choline transporters is not mandatory for ACh production, in several murine nonneuronal tissues, expression of a choline transporter-like protein (CTL-1) was recently described (47, 121). However, in these studies, expression of CTL-1, which mediates choline uptake, was not necessary for ACh production. Hence,
the requirement, if any, for active transport of choline into nonneuronal cells for ACh production remains to be determined.

**ACh Production**

ChAT (EC 2.1.3.6) is the primary enzyme that catalyzes both neuronal and nonneuronal ACh production (Figs. 2 and 3). Expression of ChAT in nonneuronal cells and tissues was reported more than 70 years ago (9). Since that initial report, ChAT expression has been identified broadly in nonneuronal cells; an incomplete list includes expression in skin keratinocytes (54, 75), lung epithelial cells (75, 115), intestinal epithelial cells (25, 75), erythrocytes (63), platelets (110), spermatozoa (11, 110), placenta and cornea epithelium (110).

**Alternative (Non-ChAT) Mechanisms of ACh Production**

ACh can be produced by a mechanism involving carnitine acetyltransferase (CrAT; EC 2.3.1.7), which is expressed in mammalian heart, skeletal muscle, retina, and urothelium (81, 109, 122, 123, 131). CrAT is a member of the carnitine acyltransferase family of enzymes that have substrate preferences for fatty acids and catalyze the exchange of acyl groups between carnitine and CoA (10). Recently, the structure of CrAT was explored and the importance of His343 in the active site was identified (69). While the impact of CrAT activity on the intracellular CoA pool is clear, its role in catalyzing ACh formation, using choline as substrate, is not well understood (95, 97). Using bromoacetylcarnitine, a CrAT inhibitor, Tucek (122) demonstrated 90% reduction in ACh production by homogenates of denervated rat muscle. In this system, ACh production was dependent on cellular choline concentration (122). Human urothelium also produces ACh (137), and, in this tissue, immunostaining and PCR demonstrated expression only of CrAT; ChAT was not detected (81). Hence, by using specific inhibitors, small interfering RNA (siRNA) knockdown, or other experimental approaches in a particular cell or tissue, it is important to clarify the relative contributions of ChAT and CrAT to nonneuronal ACh production.

Nonenzymatic ACh production in the presence of imidazoles has also been described both in vitro and in human cerebrospinal fluid (4, 19, 36). Burt and Silver (19) reported that imidazoles can mediate nonenzymatic time-, pH-, and concentration-dependent ACh production. It was proposed that ACh production by this mechanism involves formation of an N-acetylimidazole intermediate (19). In mammals, imidazole-containing molecules include creatinine and histamine. However, little is known regarding the tissue distribution, efficacy, kinetics, or frequency of this proposed mechanism of ACh production. As discussed below, collectively these considerations highlight the limitations of using ChAT expression alone as a surrogate marker of ACh production.

Finally, although the specificity of ChAT for choline is well characterized, the source of acetyl group donors can vary (129). For example, rat brain ChAT has the same affinity for acetyl-CoA, propionyl-CoA, and butyryl-CoA (106). Thus it is possible that, in addition to ACh, ChAT catalyzes synthesis of other molecules (e.g., propionyl- and butyrylcholine). On the basis of these additional observations, we conclude that expression of ChAT is neither necessary nor specific for de novo ACh production.

**Mechanisms of Nonneuronal ACh Release**

In central and peripheral nerve terminals, ACh synthesized by ChAT from acetyl-CoA and choline is translocated and stored in synaptic vesicles by vesicular ACh transporters (e.g., VAChT) (Fig. 2). After membrane depolarization, ACh-containing vesicles release ACh by exocytosis (Fig. 2). The VAChT gene is located within the ChAT gene (40). This configuration of the cholinergic gene locus (located on chromosome 10 in humans) indicates that cells expressing components of ACh production (i.e., ChAT), are also likely to express mechanisms for ACh release (i.e., VAChT).

In contrast, the mechanism underlying ACh release by normal and neoplastic nonneuronal cells is poorly understood. In cell lines derived from human small cell lung cancer (SCLC), robust expression of ChAT and VAChT, along with ACh release, is reported (115, 117). Vesamicol, a VAChT inhibitor, attenuated ACh release and proliferation of lung cancer cells (115). Nonetheless, since vesamicol did not completely inhibit ACh release from these cells, the existence of additional, nonvesicular mechanisms for ACh release is possible.

Nonneuronal expression of VAChT is also reported in rat bronchial goblet and neonatal rhesus monkey bronchial epithelial cells (80, 96, 115). However, to establish neuronlike ACh release, coexpression of ChAT and VAChT in the same cell type must be demonstrated. In contrast to demonstration of ChAT expression in a variety of nonneuronal mammalian cells, there is little evidence in nonneuronal cells for the presence of both VAChT expression and vesicular storage of ACh. These observations suggest that alternative ACh-release mechanisms are likely.

In this regard, Wessler et al. (130) demonstrated the presence of VAChT-independent release of ACh by human placenta. ACh release from placental villi was attenuated by organic cation transporter (OCT) inhibitors. OCT, members of the SLC22 and multidrug and toxim extrusion (MATE) family, mediate excretion and distribution of endogenous organic cations, xenobiotics, toxins, and environmental waste products. In human and rat bronchial epithelium and human urothelium, OCT types 1–3 are localized to the luminal membrane of ciliated epithelial cells that also express ChAT (80, 81). Moreover, OCT and VAChT have similar structural topology; 12 transmembrane domains with intracellular NH2- and COOH termini (77, 94).

Using Xenopus oocytes, transfection experiments indicate that human and murine OCT-1 and -2, but not OCT-3, are capable of transporting ACh (80). In contrast, in human placental villi, Wessler et al. (130) demonstrated that anti-sense oligonucleotides for both OCT-1 and OCT-3 reduced ACh release, whereas anti-OCT-2 oligonucleotides had no effect. These data suggest that members of the OCT family play a tissue-dependent role in nonneuronal ACh release.

Human colon cancer cells also express ChAT and produce and release ACh (25). Moreover, in surgical specimens, compared with adjacent normal tissue, colon cancer cells demonstrate increased ChAT expression (25). However, mechanisms regulating ACh release from colon cancer cells, including the role, if any, of vesicular transporters, are presently undefined. In an RT-PCR analysis of 50 cancer cell lines, colon cancer cells demonstrated expression of OCTs capable of transporting
Muscarinic Receptor Signaling by Bile Acids

Deoxycholic and lithocholic acids and their glycine and taurine conjugates interact functionally with muscarinic receptors (22, 98–100). In studies using colon cancer cell lines, these bile acids stimulated cell proliferation by a mechanism involving matrix metalloproteinase (MMP)-mediated release of an EGF receptor (EGFR) ligand, and consequent post-EGFR cell signaling (Fig. 3) (24, 26).

H508 colon cancer cells, the primary in vitro model for these studies, derive from a moderately well-differentiated adenocarcinoma of the proximal colon (cecum). Cecal conjugated bile acids, determined by enzymatic assay and gas chromatography-mass spectrometry in recently deceased humans, achieved levels that stimulate colon cancer cell proliferation in vitro (10–100 μM) (60). Because of postmortem bacterial hydrolysis, it is likely that in these studies concentrations of conjugated bile acids were underestimated. Cecal bile acid concentrations in persons with ileal disease, ileal resection, or colon cancer are not known. Certainly, depending on the severity and extent of injury, it is likely that ileal disease results in increased bile acid concentrations in the proximal colon, the area in which bile acids are most associated with increased cancer risk. Additional factors increase the likelihood that bile acid interaction with muscarinic receptors plays an important role in colon carcinogenesis: 1) fecal bile acids are in contact with colon epithelium for many years [the average age for developing colon cancer is >50 yr (133)]; 2) bile acids lack an ester linkage and are not hydrolyzed by tissue cholinesterases that rapidly inactivate ACh (23); 3) lipophilic lithocholic acid derivatives have access to muscarinic receptors in the lipid bilayer of colon cancer cell membranes (23); 4) neoplastic cells commonly lose cell membrane polarity, thereby leading to expression of receptors usually restricted to the basolateral membrane on the apical membrane; and 5) colon cancers have increased tight junction permeability, thereby providing access of luminal molecules to basolateral membrane receptors (112).

PROPOSED CRITERIA TO VALIDATE MUSCARINIC RECEPTOR-MEDIATED ACTIONS AND/OR NONNEURONAL PRODUCTION OF ACh IN CANCER

Muscarinic signaling is described in the four most common malignancies of men and women in the United States; breast, prostate, lung, and colon cancer. As shown in Table 1, muscarinic receptor expression is reported in tissues and cell lines derived from cancer of the brain (58), breast (41), colon (25),...
ACh is produced nonneuronally and that it interacts in an demonstrating ChAT expression alone is insufficient to estab-
lish that ACh acts as a local trophic factor. As discussed above, limiting choline availability or inhibiting ChAT activity should attenuate the proposed muscarinic action. Conversely, using ChE inhibitors, like eserine, to increase tissue concentrations of ACh, should augment the proposed action.

Data regarding muscarinic receptor signaling in cancer (Table 1) indicate that, for the most part, few investigations have taken a sufficiently rigorous experimental approach. There is heterogeneity in the literature regarding methods and criteria used to confirm the importance of muscarinic signaling. This is particularly true for studies proposing that cancers produce ACh that acts as a local trophic factor. As discussed above, demonstrating ChAT expression alone is insufficient to establish that ACh is produced by a cell or tissue. To confirm that ACh is produced nonneuronally and that it interacts in an autocrine and paracrine manner with cancer cells in vivo, at least two criteria must be met: 1) demonstration that a specific biological action is regulated by ACh and 2) measurement of ACh production in the tissue of interest. We propose that the following minimal criteria must be met to validate muscarinic receptor-mediated signaling and nonneuronal ACh production in cancer (Table 2).

Confirm That Muscarinic Receptor Agonists Regulate Proposed Actions

Criterion 1: demonstrate that muscarinic receptors are expressed in the tissue of interest. Expression of muscarinic receptor subtypes (M₁–R–M₃-R) can be confirmed using immunohistochemistry, in situ hybridization, immunoblotting, and RT-PCR. Nonetheless, each of these experimental approaches has potential pitfalls. For example, immunohistochemistry and immunoblotting are dependent on the specificity of muscarinic receptor antibodies. In situ hybridization is dependent on expression of sufficient message (mRNA) for detection. The presence of muscarinic receptors can also be demonstrated using a radioligand binding approach (N-methyl-[³H]scopolamimne is commonly used as a muscarinic receptor radioligand). Combining radioligand binding and immunoprecipitation using subtype-selective antibodies can detect expression of receptor subtypes with a decreased likelihood of nonspecific binding. Using any of these approaches, it is important to include appropriate positive and negative controls.

Criterion 2: muscarinic receptor agonists mimic the proposed biological action. For these studies, ACh and carbam-


Table 2. Criteria to validate MR-mediated actions (A) and nonneuronal production of ACh (B)

A. Confirm that MR agonists regulate biological actions
   1) MR are expressed in tissue of interest
   2) MR agonists mimic proposed biological actions
   3) Inhibiting ACh production attenuates proposed actions
   4) Inhibiting ACh degradation enhances proposed actions
   5) Reduced MR expression or activation attenuates proposed actions

B. Establish nonneuronal production of ACh in a tissue or cancer
   1) Demonstrate expression of choline or carnitine acetyltransferase
   2) Demonstrate production of ACh without neuronal contamination
   3) Measure ACh concentrations that mimic proposed biological actions
Establish Nonneuronal Production of ACh in a Tissue or Cancer

Criterion 1: demonstrate that the machinery needed to produce ACh exists in the tissue of interest. ChAT is the most common enzyme that catalyzes ACh production. However, de novo ACh production may exist in tissues devoid of ChAT expression or activity, provided that an alternate enzymatic or nonenzymatic system, like CrAT or imidazoles, respectively, is identified and shown to produce ACh. To identify enzymes involved in ACh production, Tucek (122) used inhibitors of ChAT and CrAT (i.e., bromo-ACh and bromoacetylcarbinine, respectively). However, these inhibitors are not substrate specific and inhibit both ChAT and CrAT. Demonstrating expression of ChAT and CrAT mRNA and protein by RT-PCR and immunostaining/immunoblotting, respectively, is required. Moreover, in mammalian tissues the extent of nonneuronal distribution of these enzymes is unknown. Hence, once the expression of ChAT or CrAT is demonstrated, criteria 2 and 3 below must be fulfilled to establish functional significance. Imidazole-mediated nonenzymatic ACh synthesis is the least understood mechanism of ACh production. Imidazole concentrations required to mediate ACh production as well as absence of both ChAT and CrAT are necessary to confirm the importance of this nonenzymatic system.

Criterion 2: demonstrate ACh production in the tissue of interest without contamination by neuronal ACh. In earlier studies, the primary methods for ACh detection used bioassays evaluating the ability of a test substance (e.g., tissue extract) to modulate contraction of frog rectus abdominis or a similar muscle preparation. Attenuation of these actions in the presence of an inhibitor, usually atropine, was considered sufficient evidence that ACh was present in the test material. It is evident that this approach is fraught with numerous potential pitfalls. Because other bioactive agents may contaminate tissue extracts and neuronal elements may be present, demonstration of such biological effects is non-specific. It is preferable to measure ACh directly in these tissues or cells using high performance liquid chromatography with electrochemical detection (HPLC-ED), scintigraphy, chromatography or other specific methods.

In tissue specimens, care must be taken not to confound results with ACh produced by neural tissue. If neural tissue is present in the test specimen, measured ACh deriving from neurons may result in a fallacious conclusion regarding nonneuronal production of ACh. Several approaches can be used to confirm nonneuronal ACh production. These include coimmunostaining with markers specific for neural tissue and ChAT to confirm that ChAT is expressed in nonneuronal cells. Laser capture microdissection may be particularly useful to avoid tissue contamination by neurons or other tissues (12). Using this technique, a cluster of cancer or other cells of interest can be removed from the tissue and any contaminating neural elements. This cluster of cells can then be used to amplify RNA or for other specific methods that will demonstrate expression of ChAT and other molecules needed for ACh production and release. Measuring ACh production by epithelial cell lines avoids confounding contamination by neuronal ACh.

Criterion 3: demonstrate that nonneuronal ACh produced at physiological concentrations elicits the proposed biological action. For a given biological action, physiological concentrations of ACh may be uncertain; in most experiments this is estimated from stimulatory doses of ACh or analogs. ACh, measured by methods described above (criterion 2), should achieve concentrations that stimulate the proposed biological action. An alternative approach is to block basal activity with muscarinic receptor inhibitors. For example, Cheng et al. (25) showed that basal proliferation of H508 colon cancer cells is inhibited by adding increasing concentrations of nonselective and M3R-selective muscarinic receptor inhibitors (25). It is inferred that H508 cells produce and release a sufficient quantity of ACh to stimulate basal levels of cell proliferation. Similarly, in SCLC cells, Song et al. (115) showed that muscarinic receptor antagonists inhibit unstimulated cell proliferation.

As shown in Table 3, these criteria have been fulfilled for few human cancers.

MUSCARINIC RECEPTOR EXPRESSION AND ACTIVATION IN CANCER

To date, in pancreatic, gastric, ovarian, cervical, and Merkel cell cancer, only the expression of muscarinic receptors has been reported (Table 1). A specific biological function following activation of these receptors has not been identified. However, in ovarian cancer, expression of muscarinic receptors is associated with reduced survival (93). In gastric cancer, ERK signaling is reported following muscarinic receptor activation. However, the failure of ERK signaling to stimulate gastric cancer cell proliferation raises questions regarding the importance of this observation (76). In human leukemia cell lines, expression of muscarinic receptors, ChAT, and CrAT, as well as ACh production, is reported (46, 72). Muscarinic receptor activation results in increased intracellular calcium and upregulation of c-fos (72). Although it is likely that these effects modulate leukemic cell function, evidence for this has yet to be reported.

As described below, more robust evidence supporting a key role for muscarinic receptor expression and activation exists for brain, breast, colon, skin, lung, and prostate cancer (Table 1).

Brain Cancer (Astrocytoma)

Muscarinic receptors (M2R, M3R, and M5R) are expressed in astrocytoma, a tumor of nonneuronal glial cells. Activation of M3R by cholinergic agonists stimulates proliferation of a primary astrocytoma cell line (58). It is proposed that proliferative actions stimulated by M3R activation are mediated by ERK and NF-κB signaling, which activate protein kinase C-ε and -ζ, respectively (56, 57, 59).

Breast Cancer

Breast cancer cell lines express muscarinic receptors that regulate cell proliferation and angiogenesis (41). Espanol et al. (42) concluded that M3R and M5R are involved in angiogenesis, whereas M1R, M2R, and M4R are involved in cell proliferation. Proliferation of breast cancer cells is regulated by postmuscarinic receptor activation of ERK signaling (68). In rats, Cabello et al. (21) showed that treatment with AChE inhibitors increased breast cancer risk. Studies using breast cancer cell xenografts treated with muscarinic agonists and antagonists indicated a role for nonneuronal ACh production in angiogenesis. However, compared with the use of other in vivo models to study the role of muscarinic receptors and ligands,
xenograft models have limitations. For example, because breast cancer xenografts were treated with test agents before implantation, this did not exactly mimic effects on breast cancer cells in vivo. Another shortcoming of xenografts is that cell lines are maintained in vitro for years. Because of selection pressures, after passage for many generations effects on cancer cell lines may no longer be representative of the original tumor. Moreover, cells in culture lack the architectural and cellular complexity of in vivo tumors, which include inflammatory cells, vasculature, and stromal components.

Colon Cancer

Colon epithelial cells express M1R and M3R (101, 136). M3R are also expressed in colon cancer cell lines, and activation of these receptors stimulates cell proliferation that is inhibited by atropine (27, 43, 44). Using eserine to inhibit ACh degradation results in potentiation of cell proliferation, whereas reducing ACh production with a choline transport inhibitor, hemicholinium-3, attenuates proliferation (25). As shown in Fig. 3, in colon cancer cells, agonist binding to M3R results in MMP-7 activation, which cleaves pro-HB-EGF, thereby releasing HB-EGF, an EGFR ligand (26). Post-EGFR signaling, mediated by ERK activation, stimulates cell proliferation (24, 27). Downstream of EGFR, activation of an additional signaling pathway involving phosphatidylinositol 3-kinase/Akt mediates effects on cell proliferation and cell survival (25, 102). In human colon cancer cells, we recently showed that muscarinic ligands exert antiapoptotic actions that are mediated by an Akt- and NF-κB-dependent mechanism (111). Collectively, these effects of muscarinic receptor activation can stimulate colon cancer cell proliferation, survival, migration, and promote angiogenesis (Fig. 3).

Melanoma

Melanoma is a skin cancer with a high metastatic potential that arises from melanocytes. Expression of all five muscarinic receptor subtypes is reported in melanoma (108, 128). In human primary melanoma cells and cells derived from metastases, expression of M3R was demonstrated by immunohistochemistry (92). Cell migration and chemotaxis play pivotal roles in metastatic spread. In normal keratinocytes, M3R activation stimulates, whereas M4R activation inhibits, migration. Muscarinic receptor inverse agonists and antagonists at-

Lung Cancer

In the United States, lung cancer is the most common lethal malignancy. Whereas SCLC cells are reported to express all five muscarinic receptor subtypes, squamous cell cancer (SCC) reportedly expresses only M1R, M3R, and M4R (114, 115). ACh and analogs stimulate lung cancer cell proliferation by interacting with M3R, thereby activating ERK and Akt signaling. Muscarinic receptor inverse agonists and antagonists attenuate this effect. In SCLC cells, augmented cell proliferation is observed following addition of eserine, and attenuated proliferation is observed with hemicholinium-3 (115). Although animal models of lung cancer, including SCLC, are described (88), in vivo studies to validate the role of muscarinic signaling in lung cancer were performed only using tumor xenografts. As discussed above, studying xenografts is not equivalent to using models of de novo cancer formation but technical limitations with de novo models necessitate the use of lung cancer xenografts. In SCLC xenografts, darifenacin, an M3R-selective antagonist, attenuated tumor growth (116).

Recently, Song et al. (114) reported that ACh production is upregulated in response to nicotinic stimulation. While darifenacin inhibited nicotine-induced proliferation of tumor cells in vitro, it attenuated SCC xenograft growth. Curiously, the investigators failed to demonstrate that muscarinic agonists induce tumor cell proliferation. Nevertheless, these experiments raise provocative questions regarding the potential for cross talk between nicotinic and muscarinic pathways in mediating lung cancer cell proliferation.

Prostate Cancer

Worldwide, in men, prostate cancer is the most common neoplasm. In prostate adenomas, Lepor and Kuhar (79) initially described and Ruggieri et al. (107) classified muscarinic receptors. Muscarinic receptors are expressed in both rat and human prostate cancer cell lines (8, 84). In 1997, Rayford et al. (104) reported that carbachol stimulates prostate cancer cell proliferation, an action attenuated by addition of muscarinic receptor inhibitors. On the basis of the pattern of inhibition with different muscarinic receptor antagonists, these investigators concluded that M3R mediate prostate cancer cell proliferation (104).

As discussed above, muscarinic receptor agonists commonly stimulate cancer cell proliferation. Nonetheless, there is a paucity of information regarding the role of muscarinic receptors in cell cycle regulation. In quiescent NIH 3T3 cells transfected with M3R, stimulation with carbachol results in ERK activation, expression of G1-phase cyclin-D1, and increased Rb phosphorylation, changes leading to DNA synthesis (90). Similarly, stimulation of normal human keratinocytes with carbachol upregulates cyclin-D1 (5). However, in activated NIH 3T3 and SCC-9 small cell lung cancer cells, muscarinic receptor stimulation causes transient cell cycle arrest and decreased proliferation (90, 132). These conflicting observations indicate that the results of muscarinic receptor activation on the cell cycle depend greatly on the cell model used and the cellular environment.

Table 3. Application of criteria in Table 2 to proposed nonneuronal ACh production in human cancers

<table>
<thead>
<tr>
<th>Organ</th>
<th>ChAT Expression</th>
<th>ACh Production</th>
<th>Physiological ACh Concentration</th>
<th>Reference</th>
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<tr>
<td>Colon</td>
<td>X X X</td>
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<tr>
<td>Lung</td>
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<tr>
<td>Small cell</td>
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<td>Squamous cell</td>
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<td>114</td>
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<tr>
<td>Pancreas</td>
<td>X ND</td>
<td></td>
<td>116</td>
<td></td>
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<tr>
<td>Skin (melanoma)</td>
<td>X ND</td>
<td></td>
<td>108</td>
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<tr>
<td>Uterine cervix</td>
<td>X ND</td>
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<td>116</td>
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</table>

ChAT, choline acetyltransferase; X, experimental evidence.
Summary

As reviewed above, ACh production is reported in a variety of normal and cancer cells (Table 1). Nonetheless, to date, strong evidence supports ACh production only by colon and lung (both small cell and squamous cell) cancer cells (25, 114, 116) (Table 2 and Table 3). In these cancer cells, muscarinic receptor antagonists inhibit basal cell proliferation, thereby providing further evidence that ACh is a growth factor. Although human keratinocytes and normal breast epithelial cells are reported to produce ACh, this phenomenon has not been described in cancers deriving from these cell types (54, 75, 128).

FUTURE DIRECTIONS

This review summarizes recent advances in elucidating the role of muscarinic receptors and ligands in cancer. Mechanistic observations regarding the role of muscarinic receptors derive primarily from in vitro investigation using cancer cell lines. Recent in vivo translational studies using lung cancer xenografts in nude mice (114, 116) and muscarinic receptor deficiency in mouse models of colon cancer (101), provide a useful framework to explore anti-neoplastic therapies targeted at mechanisms that underlie muscarinic receptor expression and activation, and ACh production and release.

Nonetheless, many questions persist regarding the role and mechanisms of muscarinic receptor activation in cancer. In colon cancer, although murine and human colon epithelial cells express both M3R and M1R, investigation has focused exclusively on M3 muscarinic receptors (3, 101). Although in mice M3R gene ablation reduces colon tumor number and size (101), the role of other muscarinic receptors, particularly M1R, requires investigation. It is conceivable that genetic ablation or reduced activation of both muscarinic receptor subtypes will have a greater anti-neoplastic effect than observed with just M3R deficiency.

Colon epithelial cells express and colon cancers over-express M1R. A more complete understanding of mechanisms underlying transcriptional and translational regulation of muscarinic receptor expression in cancer is needed. To investigate the role of these receptors in neoplasia, the availability of transgenic M1R- and M3R-deficient mice provides a useful tool. Future studies may benefit from transgenic models with organ-specific muscarinic receptor deficiency (for example, using Cre-lox-based techniques).

Likewise, mechanisms underlying transcriptional and translational regulation of ChAT and VACHT expression require further elucidation. Although experimental evidence indicates that these molecules play a role in ACh production and release, and autocrine and paracrine stimulation of lung and colon cancer cell proliferation, the regulation of these processes is unknown. Whereas, robust evidence supports production and release of ACh by lung and colon cancer cells, ChAT is expressed in cancer cells in other organs (e.g., pancreas and uterine cervix) (Table 1). Moreover, in some nonneuronal cells, organic cation transporters may mediate VACHT-independent ACh release. Regulation of these cellular components of nonneuronal ACh production and release require further investigation.

The overall importance of muscarinic receptor signaling in promoting neoplasia and cancer progression should be a prime focus of investigation. Clinical implications are broad. If toxicity and efficacy studies of anti-muscarinic agents in animal cancer models are favorable then human trials of these agents should be considered. Using the analogy of breast cancer, where tamoxifen treatment is targeted at tumors that express estrogen receptors, cancers with up-regulated muscarinic receptor expression may identify those most likely to benefit from anti-muscarinic receptor therapy. On the other hand, pesticides (e.g., parathion and malathion) commonly used in many parts of the world inhibit AChE activity. By reducing AChE activity, chronic exposure to these and similar agents may increase tissue concentrations of ACh, thereby stimulating muscarinic receptor activation. Hence, aside from other concerns regarding their use, exposure to these environmental hazards may increase the risk of neoplasia and promote faster progression of existent cancer. If, as indicated by the present review, muscarinic receptors and ligands are major players in cancer, then the environmental impact of these possible carcinogens is an additional concern that must be explored and addressed.

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