Activated Cholinergic Signaling Provides a Target in Squamous Cell Lung Carcinoma

Pingfang Song,[1] Harmanjatinder S. Sekhon,[1] Xiao Wen Fu,[1] Michelle Maier,[1] Yibing Jia,[1] Jie Duan,[1] Becky J. Proskosil,[1] Courtney Gravett,[1] Jon Lindstrom,[2] Gregory P. Mark,[3] Saurabh Saha,[4] and Eliot R. Spindel[1]

[1]Division of Neuroscience, Oregon National Primate Research Center, Oregon Health and Sciences University, Beaverton, Oregon; [2]Department of Neuroscience, University of Pennsylvania Medical School, Philadelphia, Pennsylvania; [3]Department of Behavioral Neuroscience, Oregon Health and Sciences University, Portland, Oregon; and [4]Novartis Institutes for Biomedical Research, Cambridge, Massachusetts

Abstract

The binding of exogenous nicotine to nicotinic acetylcholine (ACh) receptors (nAChR) and the binding of endogenous ACh to both nAChR and muscarinic ACh receptors (mAChR) stimulate growth of both small cell and non–small cell lung carcinomas. Understanding how cholinergic signaling is up-regulated in lung cancer may suggest new therapeutic approaches. Analysis of 28 squamous cell lung carcinomas (SCC) showed increased levels of α5 and β3 nAChR mRNA and increased levels of ACh associated with increased levels of choline acetyltransferase mRNA and decreased cholinesterase mRNAs. Lynx1, an allosteric inhibitor of nAChR activity, was also decreased in SCC. Thus, cholinergic signaling is broadly increased in SCC caused by increased levels of receptors, increased levels of ligands, and decreased levels of receptor inhibitors. Partially explaining the cholinergic up-regulation seen in SCC, incubation of the H520 SCC cell line with nicotine increased levels of ACh secretion, increased expression of nAChR, and, as measured by electrophysiologic recording, increased activity of the expressed nAChR. Consistent with these effects, nicotine stimulated proliferation of H520 cells. One approach to blocking proliferative effects of nicotine and ACh on growth of lung cancers may be through M3 mAChR antagonists, which can limit the activation of mitogen-activated protein kinase that is caused by both nicotinic and muscarinic signaling. This was tested with the M3-selective muscarinic antagonist darifenacin. Darifenacin blocked nicotine-stimulated H520 growth *in vitro* and also blocked H520 growth in nude mice *in vivo*. Thus, cholinergic signaling is broadly up-regulated in SCC and blocking cholinergic signaling can limit basal and nicotine-stimulated growth of SCC. [Cancer Res 2008;68(12):4693–700]

Introduction

Lung cancer is the number one cause of cancer death in the United States with deaths in 2007 estimated to exceed 160,000 (1). Lung cancer is classified into small cell lung carcinoma (SCLC), which accounts for approximately 14% to 20% of cases, and non-SCLC (NSCLC), which accounts for the remaining cases. NSCLC consists primarily of squamous cell carcinoma (SCC) and adenocarcinoma. Despite improvements in responses to increasingly sophisticated combination of surgery, radiation, and chemotherapy (2), survival remains low. Thus, the development of new therapeutic approaches is clearly needed.

Smoking is associated with the vast majority of lung cancer (3). Although the primary mechanism of smoking-induced carcinogenesis derives from carcinogens in smoke, recent data show that nicotine and nitrosamines in smoke bind to nicotinic acetylcholine (ACh) receptors (nAChR) on lung cancers to stimulate growth and potentially tumor progression (4–9). Expression of nAChR is seen in both SCLC and NSCLC (4, 10–12). Expression of nAChR in lung cancers derives from expression of nAChR in normal lung cells. Normal bronchial epithelial cells express nAChR as part of a cholinergic autocrine loop (13–16) in which all proteins needed for cholinergic signaling are present, including the ACh-synthesizing enzyme choline acetyltransferase (ChAT), the vesicular ACh transporter (VAChT), the high-affinity choline transporter CHT1, the ACh hydrolyzing enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), nAChRs, and muscarinic ACh receptors (mAChR). We and others have also shown that the interaction of ACh with both nAChR and mAChR stimulates cell proliferation (11, 17–20), and we have previously reported that SCLC similarly express this cholinergic autocrine loop and that muscarinic antagonists can inhibit SCLC cell proliferation.

Whereas the expression of nAChR in SCC has been described, the expression of the complete cholinergic autocrine loop in SCC has yet to be described. In this article, we show that SCC, like SCLC, expresses a cholinergic autocrine loop. Lam and colleagues (10) recently reported in a series of NSCLC (predominantly adenocarcinomas) that some nAChRs are changed in tumor relative to normal lung and that nicotine exposure seems to change receptor expression. As we show here, changes in cholinergic signaling in SCC are not limited just to nAChR expression but large changes in ACh synthesis and degradation are also present. Given the up-regulation of cholinergic signaling in lung cancer, the role of smoking and continued stimulation of cancer cells by nicotine is of obvious importance. The interaction of nicotine with its receptor can lead to receptor inactivation or receptor activation depending on receptor subtype and cell type. Here, we show that nicotine up-regulates both nAChR expression and activity.

West and colleagues (4) and Tsurutani and colleagues (21) have shown Akt activation by nicotine, and our lab and other laboratories have also shown mitogen-activated protein kinase (MAPK) activation by nicotine (6, 22–24). Endogenous ACh released from lung cancers signals through both nAChR and

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Eliot R. Spindel, Division of Neuroscience, Oregon National Primate Research Center, Oregon Health and Sciences University, 505 Northwest 185th Avenue, Beaverton, OR 97006. Phone: 503-690-5512; Fax: 503-690-5384; E-mail: spindele@ohsu.edu.

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mAChR and also leads to Akt and MAPK activation (22). Because MAPK is activated by both nicotinic and muscarinic pathways and we have previously shown that M3 muscarinic antagonists decrease basal MAPK activation as well as blocking ACh-induced MAPK and Akt, M3 muscarinic antagonists present a potential approach to blocking both nicotinic and muscarinic proliferative pathways. In this article, we also show that muscarinic M3 antagonists can block basal proliferation of SCC as well as nicotine-stimulated SCC proliferation.

Materials and Methods

SCC cell lines and SCC samples. The SCC cell line H520 [American Type Culture Collection (ATCC)] was used for most studies and maintained as recommended by ATCC. For analysis of expression of cholinergic RNAs and Western blot analysis, 28 frozen archival SCC samples were obtained from the tumor bank of the Department of Pathology of the Oregon Health and Science University (OHSU). For 6 of the 28 samples, adjacent normal tissue was also available. H&E-stained slides of the samples were reviewed to confirm diagnosis and establish degree of differentiation. For immunohistochemical analysis of cholinergic gene expression, another 31 archival, paraffin-embedded SCC samples were obtained from the OHSU Department of Pathology. For high-performance liquid chromatography (HPLC) analysis of ACh content of SCC, an additional eight SCC and eight normal lung samples were obtained form the OHSU tumor bank. An additional six SCC cell lines (H647, H1703, H1869, H2066, HTB-58, and HTB-59) from ATCC were also screened for cholinergic components and responses.

Real-time PCR and Western blots. Real-time PCR was used to quantify cholinergic gene expression in tumor samples as previously described (11, 25). Total RNA and protein in SCC and the normal lung tissues were prepared with TRI-reagent (Molecular Research Center, Inc.; ref. 26). The probes and primers used in real-time PCRs were as described previously (11) or are listed in Supplementary Table S1. All reactions were run in triplicate and RNA levels were normalized to 18S RNA expression as an internal standard. In addition, no-reverse transcriptase controls were run with all RNAs to check for genomic DNA contamination. Western blot analysis was performed as described previously (25) using actin as an internal standard. Antibodies are as described below for immunohistochemistry.

Immunohistochemistry. Immunohistochemistry was performed as previously described (11, 15, 22). Antibodies used were anti-ChAT (MAB305); anti-AChE (MAB337) from Chemicon International, Inc.; anti-VAChT (H-V005) from Phoenix Pharmaceuticals; anti-CHT1 generously provided by R. Blakely (Vanderbilt University, Nashville, TN; ref. 11); and anti-Lynx1 as previously described (25). Muscarinic receptor antibodies were as follows: anti-M2 (WR-3791) from R&D Systems and anti-M3 (H210) from Santa Cruz Biotechnology (11, 15, 25). Nicotinic receptor antibodies were as follows: anti-α3 (mAb356), anti-α4 (mAb299), anti-α7 (mAb319), anti-β2 (mAb3724), and anti-β4 (mAb337) as described by Song and colleagues (11) and Sekhon and colleagues (15) and anti-β3 from Santa Cruz Biotechnology. Immunostaining was independently scored by two readers on a scale from 0 to 4, where 0 = no staining, 1 = focal weak staining, 2 = diffuse weak staining or focal strong staining, 3 = diffuse medium staining, and 4 = diffuse strong staining. The readers were blinded to each other's results and other reads on the slides.

ACh assay. ACh synthesis by SCC was assayed by HPLC as described previously (11). For measurement of ACh in tissue, 100 to 200 mg of samples were homogenized in 2 mL of 90% methanol containing 0.1 mol/L formic acid and then centrifuged at 10,000 rpm for 15 min. Supernatant (1 mL) was lyophilized and stored at −80°C. The dried samples were dissolved in 80 to 120 μL of the HPLC mobile phase and filtered and 10 μL were injected into the HPLC. Each sample was measured at least in duplicate. Standard ACh (2 nmol/L) was added to 2 mL 90% methanol and processed as above for determination of ACh recovery. For measurement of ACh secretion by SCC cell lines, 500,000 cells in 1 mL fresh medium were plated into each well of

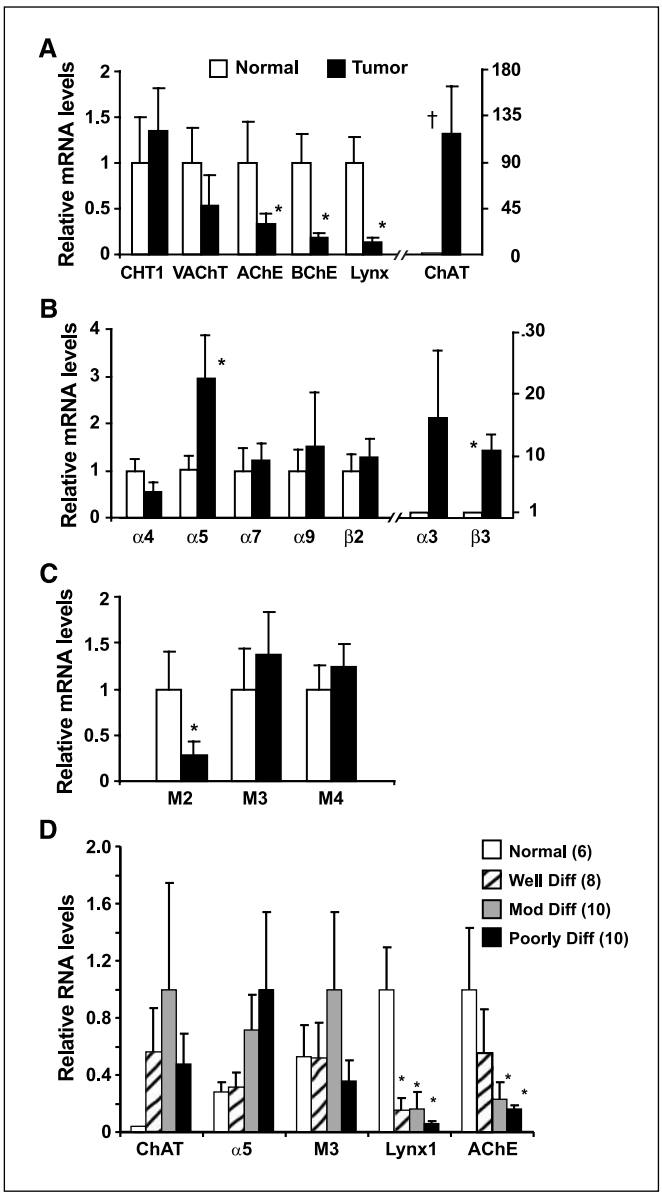

Figure 1. Real-time PCR analysis of cholinergic mRNA expression in SCC tumor (*black columns*) versus adjacent normal lung (*white columns*). Real-time PCR was performed as described in Materials and Methods using 18S RNA as an internal standard. RNA levels were normalized to controls to allow comparison of fold change between tumors and normal. For ChAT, α3, and β3, fold change is plotted on right axes. *A,* expression of ChT1, VAChT, AChE, BChE, Lynx1, and ChAT mRNA in SCC and adjacent normal lung tissues. *, $P < 0.03$, expression of AChE, BChE, and Lynx1 was significantly lower in tumors than adjacent normal tissue; †, $P < 0.01$, expression for ChAT was significantly higher in tumors than normal. *B,* expression of nAChR subunits in SCC. mRNA levels for α5 and β3 nAChR subunits were significantly higher in tumors than normal (*, $P < 0.025$); other differences were not significant. *C,* expression of muscarinic receptors in SCC and adjacent normal tissue. mRNA levels for M2 mAChR were significantly decreased in tumors than normal (*, $P < 0.05$); levels of M3 and M4 were unchanged. *Columns,* mean ($n = 28$ for tumors; $n = 6$ for adjacent normal lung tissues); *bars,* SE. *D,* relative RNA levels of ChAT, α5 nAChR, M3 receptor, Lynx1, and AChE in SCCs plotted according to degree of differentiation. *, $P < 0.05$, compared with normal tissue by Tukey-Kramer after one-way ANOVA. Data for each RNA are normalized to 1.0 for the highest value within each RNA to allow comparison of changes within RNAs. Number of samples of each grade is shown in figure legend in parentheses.

24-well dishes and neostigmine (5×10^{-5} mol/L; Sigma-Aldrich) was added to all dishes to inhibit ACh degradation. Twenty-four and 48 h after plating, supernatants were collected, frozen on dry ice, and stored at −80°C.

Calcium fluorometry. Changes in SCC cell line intracellular calcium concentration $[Ca^{2+}]_i$ elicited by ACh was measured fluorometrically using a FlexStation (Molecular Devices Corp.) as described previously (22). Drugs used (ACh, carbachol, mecamylamine, and atropine; Sigma-Aldrich) were diluted to desired concentrations with HBSS supplemented by 20 mmol/L HEPES plus 2 mmol/L $CaCl_2$ and applied at the concentrations shown in the results.

Electrophysiology. Cells were transferred to a recording chamber mounted on the stage of a Leica microscope and perfused with Krebs solution with the following composition (in mmol/L): 140 NaCl, 3 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 5 glucose (pH 7.3; osmolarity, 300 mOsm). Cells were perfused at 2 to 3 mL/min with bathing solution at room temperature. Drugs were applied by fast step perfusion system (SF-77B perfusion, Warner Instruments). To isolate inward currents, an internal pipette solution with the following composition was used (in mmol/L): 130 CsCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 EGTA, 10 HEPES, and 4 Mg-ATP (pH adjusted to 7.2 with CsOH). Whole-cell currents were recorded from H520 cell using standard patch-clamp techniques (27). The resistance of the recording pipettes was 3 to 5 MΩ. Cells were voltage clamped at −60 mV. A MultiClamp 700B (Axon Instruments) was used to record whole-cell currents. Voltage clamp protocols, data acquisition, and analysis were performed using DigiData 1322A interface and pClamp9 software (Axon Instruments).

Cell proliferation assay. H520 SCC cells were used to determine the ability of the M3 antagonist darifenacin to inhibit nicotine-stimulated proliferation. Cells were plated at a concentration of 2,500 per well as described above. The following day, cells were changed to medium containing 1% FCS plus drugs as described in Results. Cell density was monitored using the CellTiter-Blue assay (Promega Corp.) as previously described (22).

Nude mice studies. For tumor xenograft growth studies, male mice (*nu/nu*; Charles River Laboratories) were injected with H520 SCC tumor cells and treated with the M3 receptor antagonist darifenacin and tumor growth was monitored. Darifenacin was generously provided by Novartis Pharma AG. H520 cells were grown as described above, and 5×10^6 cells were injected s.c. into the right flank of each mouse as described previously (22). Tumors were allowed to grow for 1 wk, and then drug administration was initiated and continued for the next 4 wk. Darifenacin was dissolved in 50% DMSO/50% PBS and administered by s.c. implanted osmotic minipumps (Alzet model 2004) at doses of 6.0 mg/kg/d. Control animals received minipumps filled with 50% DMSO/50% PBS. Tumor volume was determined weekly by measuring with calipers (volume = height × width × depth). The study was terminated after 4 wk of drug administration. Ten animals were used per group. At sacrifice, tumors were removed and weighed.

Statistical analysis. Data are presented as mean ± SE. Data of real-time PCR and Western blot analysis were analyzed by *t* test using NCSS 2004 statistical software. EC_{50} and IC_{50} in the Calcium fluorometry were calculated with the SoftMax program 4.0.1 provided by Molecular Devices. Cell proliferation was analyzed by one- or two-way ANOVA followed by Tukey-Kramer multiple comparison tests.

Results

Expression of cholinergic mRNAs and proteins in SCC. Real-time PCR was performed to characterize cholinergic gene expression in 28 SCC tumors and 6 adjacent normal tissues as described in Materials and Methods. Frozen SCC samples were obtained from the OHSU tumor bank and diagnosis and histologic grade were confirmed by examination of H&E-stained sections of the same tumors. As shown in Fig. 1*A*, the enzymes necessary for the synthesis and degradation of ACh were all present in SCC. ChAT was significantly increased in SCC compared with adjacent normal tissue, whereas levels of CHT1 and VAChT were not significantly changed (Fig. 1*A*). Levels of mRNA that encode proteins that serve to limit cholinergic signaling, AChE, BChE, and Lynx1, were all significantly decreased in tumors compared with controls (Fig. 1*A*). All nicotinic receptors measured in tumors trended higher than in normal tissues and levels of α5 and β3 were significantly increased in the tumors (Fig. 1*B*). Levels of M2 mAChR mRNA were significantly decreased in tumors compared with controls, whereas significant differences between tumors and controls were not seen in levels of M3 and M4 mAChR mRNA (Fig. 1*C*). Changes in cholinergic protein levels as measured by Western blot analysis mirrored changes in RNA levels and

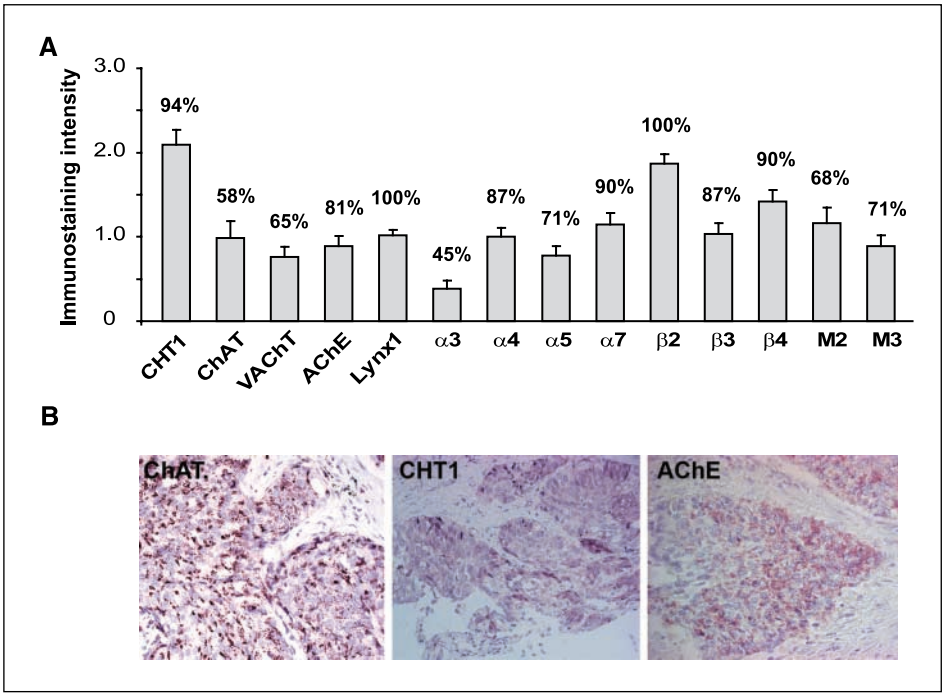

Figure 2. *A,* relative immunostaining for components of cholinergic signaling as shown. Intensity of staining scored from 0 to 4 as described in Materials and Methods and averaged for all samples. Percentage of samples with positive staining shown above each bar. $n = 31$. *B,* representative immunohistochemical staining of ChAT, CHT1, and AChE expression in SCC tumors. Magnification, ×200. Chromogen = VIP for all panels.

significant decreases in levels of AChE, M2, and Lynx1 between tumors and controls were observed (data not shown). Levels of M3 and M4 by Western blotting were not significantly different between groups (data not shown). The differences in cholinergic mRNAs between tumors and normals tended to increase as tumors became less differentiated. Levels of ChAT, α5 nAChR, and M3 mAChR trended higher in less differentiated SCC and levels of Lynx1 and AChE RNA trended lower in less differentiated SCC (Fig. 1*D*).

Next, the expression of cholinergic proteins in SCC by immunohistochemistry was determined in 31 SCC tumors (Fig. 2). As can be seen in Fig. 2*A*, consistent with the analysis of mRNA expression, the majority of SCC expressed the proteins needed to both synthesize ACh and to respond to ACh through both the nicotinic and muscarinic cholinergic pathways. Representative staining for ChAT, CHT1, and AChE is shown in Fig. 2*B*. Approximately 60% of SCC expressed ChAT by immunohistochemistry and essentially all expressed at least one subtype of nicotinic or muscarinic receptors. Thus, the majority of SCC can synthesize ACh and almost all would respond to exogenous ACh or nicotine.

In addition, as listed in Materials and Methods, seven SCC cell lines were analyzed for ChAT and cholinergic receptor expression. Similar to the tumor samples, all SCC cell lines examined expressed at least one subtype of nicotinic or muscarinic receptor and six of seven cell lines expressed ChAT (data not shown). Six of seven SCC lines examined also showed increased intracellular calcium in response to ACh challenge. Because the H520 SCC cell line proved the most amenable for patch clamp analysis, it was used for more detailed studies as described below.

ACh is elevated in SCC. Because the presence of the enzyme to synthesize ACh does not guarantee actual production of ACh, levels of ACh were assayed in eight additional tumor samples and eight normal lung samples (Fig. 3). As shown in Fig. 3*A*, high levels of ACh were present in six of eight tumor samples but nearly undetectable in all but one normal lung sample. This is consistent with the dramatic difference in ChAT mRNA levels between tumor and adjacent normal tissues shown in Fig. 1*A* and the decreased levels of cholinesterase shown in Fig. 1*D*. As shown in Fig. 3*B*, nicotine exposure may play a role in up-regulating ACh levels in tumors as incubation of the H520 SCC cell line with nicotine mildly, but significantly, increased ACh secretion by SCC cells under conditions (10% FCS) in which nicotine did not increase cell number.

Nicotine up-regulates and activates nicotinic receptors in SCC. To assess how chronic nicotine exposure affects nAChR expression and activity, H520 cells were incubated with nicotine and activity of nicotinic receptors in SCC H520 cells was measured by whole-cell voltage clamp. As shown in Fig. 4*A*, incubation in 2 μmol/L nicotine for 48 h up-regulated nAChR expression as shown by immunostaining. As shown in Fig. 4*B*, the nAChRs expressed in SCC are active with both α7 nAChR and non–α7-containing nAChR currents. Application of 100 μmol/L nicotine to a H520 cell voltage clamped at −60 mV elicited a transient inward current (Fig. 4*B*), and mean peak nicotine-induced inward current (I*nic*) was −120.4 ± 11.5 pA (n = 9). Desensitization time constant for I*nic* was 8.9 ± 3.9 s (n = 5). The EC_{50} for receptor activation by nicotine was 57 μmol/L, and the Hill coefficient was 1.1 (data not shown). Currents induced by 100 μmol/L nicotine were reversibly suppressed by 30 μmol/L mecamylamine, a nonselective nAChR antagonist, and by 20 nmol/L methyllycaconitine (MLA), a specific α7-specific nAChR antagonist (Fig. 4*B*). Nicotine-induced inward current was reduced 85 ± 5% (n = 4) by mecamylamine and 76 ± 7.6% (n = 4) by MLA.

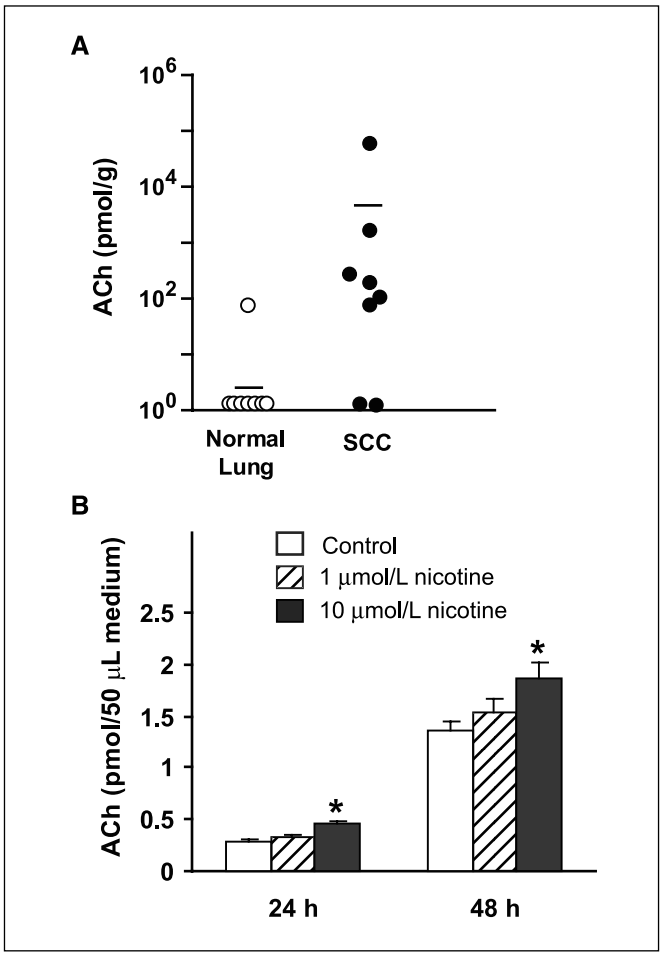

Figure 3. ACh expression by SCC tumors, normal lung, and cell lines. *A,* ACh content of normal lung and SCC tumors. ACh content of tumors expressed as pmol/g wet weight. ACh content of tumors was significantly higher than normal lung (note data plotted on log scale). *B,* nicotine increased ACh secretion by H520 SCC cells in culture. H520 cells (0.5 million) were plated in 24-well plates and incubated overnight. On the second day, fresh medium containing 50 μmol/L neostigmine and nicotine at the concentrations shown was added. After an additional 24 or 48 h of incubation, ACh content in the medium was measured. *Columns,* mean of four experiments; *bars,* SD. *, $P < 0.02$, compared with control.

These studies show that H520 cells express both functional α7 nAChR and non–α7-containing nAChR. Recording from single cells showed that chronic exposure to nicotine resulted in increased activity of the nAChR expressed in the cultured SCC cells (Fig. 4*C* and *D*). Thus, chronic nicotine exposure both up-regulates and activates nAChR expression in SCC.

We have previously reported that M3 muscarinic antagonists can block SCLC growth by targeting MAPK proliferative pathways, which are activated by both nicotinic and muscarinic cholinergic receptors. Given that SCC express similar nAChR and mAChR as SCLC, this suggests that M3 antagonists might similarly inhibit proliferation in SCC. H520 cells express functional mAChR as shown by the ability of atropine to block the ACh-induced increase in intracellular calcium (Fig. 5*A*). As shown in Fig. 5*B*, the selective M3 mAChR antagonist darifenacin blocked the nicotine-induced increase in H520 cell proliferation *in vitro*. Darifenacin also significantly inhibited growth of H520 SCC xenografts in nude mice (Fig. 5*C* and *D*). This suggests that

SCC tumor growth can be blocked by targeting the activated cholinergic pathways present in SCC.

Discussion

Lung cancer expresses an intrinsic cholinergic signaling system such that exogenous nicotine and endogenous ACh can stimulate tumor growth. As we show here, the cholinergic system in SCC is up-regulated at multiple levels. This up-regulation combined with smoking by most lung cancer patients provides not only a considerable proliferative stimuli but also a pathway to target for new therapeutic approaches to lung cancer.

In early studies, Schuller (28) showed that nicotine stimulated growth of lung cancer cell lines and Maneckjee and Minna (29) showed that nicotine blocked the inhibitory effect of opiates on lung cancer cell line growth. Subsequent studies have shown that nicotine acting through nAChR activates lung cancer growth through both the Akt and MAPK pathways (4–9). Similarly, ACh acting through mAChR as well as nAChR has been shown to lead to cell proliferation by activation of MAPK [extracellular signal-regulated kinase 1/2 (ERK1/2)] and stimulation of cell cycle progression (17, 18, 30). Studies from our laboratory showed that lung cancers express nAChR and mAChR as part of a stimulatory autocrine cholinergic pathway and that, in addition to cholinergic receptors, lung cancers synthesize and secrete ACh and express cholinesterases (11).

SCCs are derived from bronchial epithelial cells. Thus, not surprisingly, normal bronchial epithelial cells also express a cholinergic autocrine loop (13). However, as shown in Figs. 1–3, cholinergic signaling is markedly up-regulated in SCC compared with normal lung. As shown in Fig. 1*A*, ChAT is strongly up-regulated in SCC, whereas cholinesterases are down-regulated. This combination of increased synthesis and decreased degradation causes dramatic increases in ACh content of tumor compared with normal lung as shown in Fig. 3*A*. Thus, SCCs secrete increased levels of ACh to provide an endogenous proliferative stimuli to both mAChR and nAChR. The mechanism underlying the increased ChAT expression in SCC is not clear, although nicotine itself stimulates ACh secretion from H520 cells in culture (Fig. 3*B*). The observation of decreased cholinesterases in SCC is consistent with the results of Martinez-Moreno and colleagues (31) who reported decreases in AChE and BChE activity in both SCC and large cell carcinoma of the lung. The potential importance of decreased cholinesterase in tumor growth is further supported by Cabello and colleagues (32) who showed that long-term treatment of rats with cholinesterase inhibitors led to increased formation of mammary carcinomas that could be blocked by administration of the muscarinic antagonist atropine.

There is also a striking reduction in the levels of Lynx1 in SCC (Fig. 1*A*). Lynx1 is a member of a newly described family of allosteric modulators of nicotinic receptor activity (33–35). Lynx1

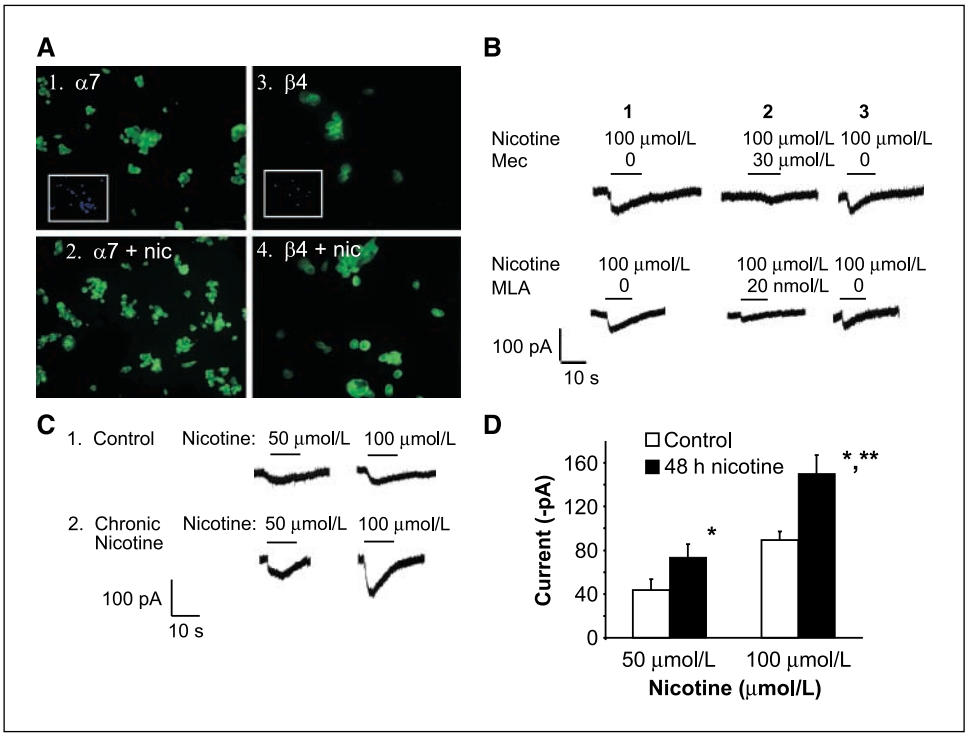

Figure 4. Effect of nicotine on nAChR activity in SCC cells. *A,* chronic nicotine exposure up-regulates α7 and β4 nAChR immunostaining in H520 cells. *1,* control α7 nAChR immunostaining in H520 cells after 48 h of incubation in medium alone. *Inset,* nonspecific staining control. *2,* increased α7 immunostaining after 48 h of incubation in medium + 2 μmol/L nicotine. α7 nAChR staining increased by 29 ± 5% (mean ± SE; $n = 3$ experiments; one or two coverslips per experiment; 15 fields of view per coverslip). *3,* control β4 nAChR staining. *4,* increased staining after 48 h of incubation in 2 μmol/L nicotine. β4 nAChR staining increased by 24 ± 6% (mean ± SE; $n = 3$ experiments; one or two coverslips per experiment; 15 fields of view per coverslip). *B,* nicotinic receptor inward currents in H520 cells. Nicotine induces inward currents in H520 cells, which are blocked by the nAChR antagonists mecamylamine and MLA. *C,* chronic nicotine exposure up-regulates responses to nicotine in H520 cells. *1,* whole-cell current in control H520 cells in response to application of nicotine by perfusion at concentrations shown (50 and 100 μmol/L). *2,* after 2 μmol/L nicotine treatment for 48 h, whole-cell currents in response to application of nicotine by perfusion at concentrations shown (50 and 100 μmol/L). Holding potential was −60 mV. Experimental data were fitted by the Hill equation with EC_{50} = 57 μmol/L and Hill coefficient = 1.1. *D,* quantitation of increase in peak currents induced by nicotine after chronic nicotine exposure. *, $P < 0.01$, for effect of nicotine by two-way ANOVA; **, $P < 0.05$, compared with control by Tukey-Kramer multiple comparison test.

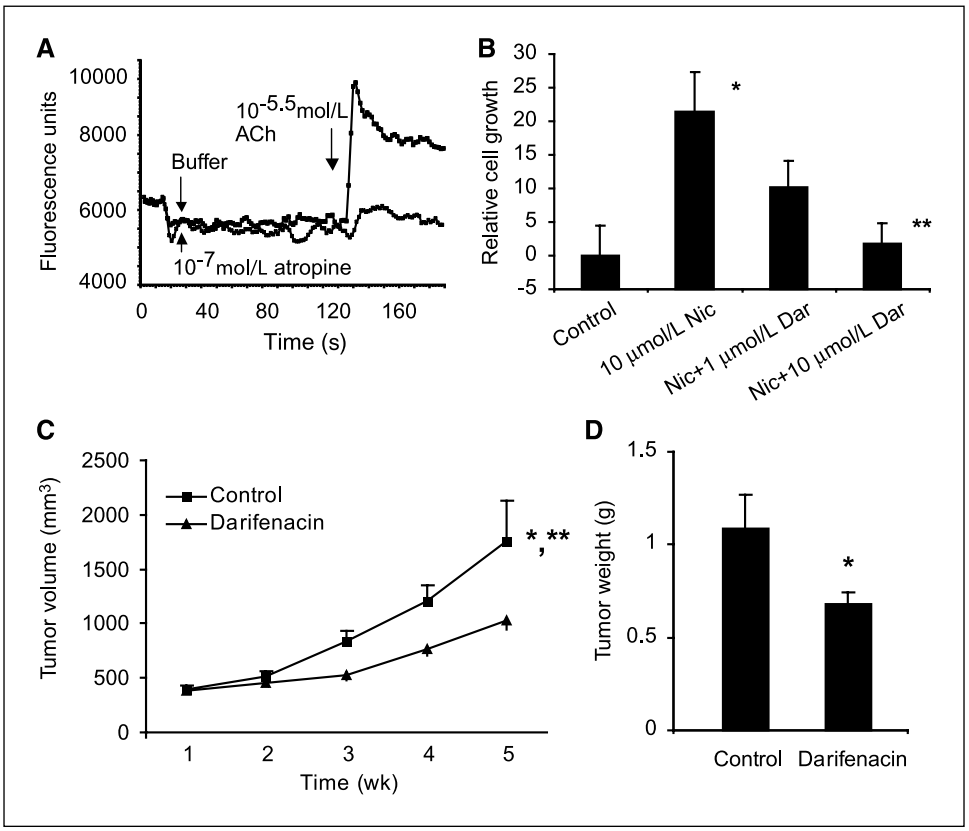

Figure 5. Muscarinic antagonists inhibit cholinergic signaling and SCC cell growth. *A,* the muscarinic antagonist atropine inhibits the ACh-induced increase in intracellular calcium in H520 cells. *B,* the M3 muscarinic receptor antagonist darifenacin inhibits the nicotine-induced increase in H520 SCC cell growth. *Columns,* mean of two separate experiments presented as % of increased cell proliferation in control; *bars,* SE. *, $P < 0.05$ versus control; **, $P < 0.05$ versus 10 μmol/L nicotine. *C,* effect of darifenacin (6.0 mg/kg/d) on growth of H520 SCC xenografts in nude mice. Tumor cells were injected on day 0 and darifenacin was started 1 wk later. *, $P < 0.001$, for overall effect of darifenacin on tumor growth by two-way ANOVA; **, $P < 0.05$, for treated versus control at 5 wk by Tukey-Kramer multiple comparison test. *D,* tumor weight at 5 wk. *, $P < 0.05$, compared with control by *t* test.

has been shown to attenuate responses to ACh and to increase the extent to which ACh and nicotine desensitize nAChR (33). In brain, Lynx1 knockout mice show dramatically increased responses to nicotine (36). Our laboratory has recently shown that Lynx1, the nAChR accessory protein, is expressed in normal bronchial epithelium (25). We now find that Lynx1 is widely expressed in SCC but is expressed at significantly lower levels in tumors than adjacent normal tissue. Thus, decreases in Lynx1 would be expected to potentiate SCC responses to exogenous nicotine and endogenous ACh.

As shown in Figs. 1 and 2, nicotinic receptors are expressed in the majority of SCC consistent with their expression in normal bronchial epithelium (25). Consistent with West and colleagues (4), nAChR subunits, including α3, α5, α7, α9, and β2, were found in most SCC (Figs. 1 and 2). M2, M3, and M4 mAChR subtypes were also found to be expressed in SCC (Figs. 1 and 2). These results imply that SCC might have homomeric nAChR containing α7 or α9 subunits and heteromeric forms containing both α and β subunits. We did not check for α10, but given its wide expression in peripheral tissue, it is also likely present in SCC and could form heteromers with α9 (37). There were no significant differences in levels of α3, α7, α9, and β2 nAChR subunits and M3 and M4 mAChR receptor between SCC and the adjacent normal tissues but levels of α5 and β3 nAChR subunits were significantly higher in tumor than normal tissues. The presence of increased levels of α5 and β3 subunits would suggest that growth of some SCC might be sensitive to mecamylamine, which is an inhibitor of heteromeric nAChR subtypes. Consistent with this, Zhu and colleagues (38) have shown that mecamylamine inhibits secondhand smoke stimulation of Lewis lung cancer cells in nude mice and we have previously shown that mecamylamine inhibits growth of SCLC cells *in vitro* (11). Interestingly, levels of M2 receptors were decreased and we have previously reported that M2 activation seems to inhibit lung cancer growth (39).

Lam and colleagues (10) recently examined expression of nAChR in a series of NSCLC. Interestingly, they did not see the increase in α5 and β3 we observed but instead saw a small decrease in α4 and β4, which we did not observe. However, their series was primarily adenocarcinomas with only 6 of 66 being SCC. In their individual results, levels of α4 and β4 did in fact seem higher in SCC than adenocarcinomas. Differences in receptor expression between lung adenocarcinomas and SCCs are to be expected because they derive from different lung cell types that also have different patterns of receptor expression. These differences show how important it is to fully characterize the different nAChR receptor expression by different lung cancer types as this may affect responses to therapy. Lam and colleagues (10) also reported a striking difference in nAChR receptor expression between adenocarcinomas from smokers and nonsmokers. SCC in never smokers is less common than adenocarcinoma in never smokers (3), so, not surprisingly, none of the SCC in the series we studied came from nonsmokers so we could not examine effects of smoking on receptor expression. In addition, we did not have pack-year histories or gender identification associated with the tumors. It is quite possible that the number of pack-years of exposure to exogenous nicotine might affect nAChR receptor expression patterns by the tumors. The role of gender on cholinergic expression patterns is also of great interest given the significantly higher frequency of lung adenocarcinomas in never smoker women than never smoker men (40).

What makes the expression of nAChR in lung cancer so significant is that the majority of patients are smokers and many continue to smoke even after diagnosis. Thus, the nAChR in lung cancers are continually exposed to nicotine. The effect of nicotine on nicotinic receptor activity is highly complex and has been extensively studied in brain. In some neurons, chronic nicotine exposure can up-regulate and inactivate nAChR (41); in other systems, chronic nicotine up-regulates and activates nAChR (42). Nicotine has also been recently shown to act as a chaperone and increase targeting of mature nAChR to the cell membrane (43). As shown in Fig. 4*A*, nicotine increased expression of nAChR in H520 SCC cells. This is consistent with the finding of Lam and colleagues (10) and consistent with our previous finding that chronic nicotine increases nAChR expression in normal lung cells (15). Importantly, the up-regulation of nAChR expression is associated with increased receptor activity as shown by whole-cell voltage clamp recordings (Fig. 4). Antagonist studies show activity of both homomeric α7 nAChR and non–α7-containing heteromeric nAChR (Fig. 4*B*).

In comparing levels of expression of components of the cholinergic signaling pathway between the normal and neoplastic lung samples, it is striking that all the significant changes were in the direction of increased cholinergic signaling in the tumors. First, levels of ACh increased in tumors reflecting increased ChAT and decreased cholinesterase expression. The increased levels of tumor ACh would then be able to stimulate proliferative pathways through both muscarinic and nicotinic mechanisms. Cholinergic stimulation of growth would likely be increased as levels of some nAChR subunits are increased, and levels of inhibitory M2 muscarinic receptors are decreased. Nicotinic receptors would also likely show less desensitization from endogenous ACh and exogenous nicotine as levels of Lynx1 are decreased. Finally, we have shown that nicotine up-regulates nAChR activity, so smoking will amplify responses to the increased levels of ACh found in lung cancers as well as amplify responses to continued smoking. Thus, the changes seen in cholinergic signaling in SCC strongly support a role for nicotine to stimulate growth and progression of the tumors. Consistent with this, continued smoking has been shown to have a negative correlation on lung cancer survival (44–46). Therefore, in smokers with lung cancer, tumors would be stimulated both by endogenous ACh and exogenous nicotine. Each by itself is capable of stimulating proliferation of tumor cells and the combination of both has the potential to provide an additive stimuli to proliferation.

The mechanisms underlying the differences in cholinergic expression between tumors and normal lung remain to be determined and require further studies. The differences may derive from the cells of origin of SCC in that expression of nAChR, mAChR, and ChAT is normally greater in bronchial epithelium than lung parenchyma. Alternately, differences may reflect the effects of nicotine-stimulating cholinergic expression in the tumor, reflect increased proliferation by the tumor, or reflect other changes related to tumorigenesis. Finally, it is possible that the differences could reflect sampling differences between normal and tumor tissue in which the normal samples do not represent a good sampling of normal large airway epithelium.

The up-regulation of cholinergic signaling in SCC suggests that this pathway can be targeted for therapeutic intervention. A model of the cholinergic pathway is shown in Fig. 6, and as can be seen, there are multiple potential targets. West and colleagues (4) have shown that Akt pathway can be effectively used to block nicotine-associated proliferation, and Trombino and colleagues (47) have shown that nAChR antagonists can inhibit growth of mesotheliomas. We have chosen to focus on the muscarinic receptor as a potential target because oral muscarinic antagonists are well tolerated and in wide clinical use for overactive bladder (48) and inhaled muscarinic antagonists are widely used for chronic obstructive pulmonary disease (49). We have previously reported that M3 muscarinic antagonists can block growth of SCLC cell lines *in vitro* and in nude mice xenografts by preventing ACh- and nicotine-induced increases in MAPK phosphorylation as well as lowering basal levels of MAPK phosphorylation. Because of the inhibitory effects of M3 antagonists on SCLC proliferation, we investigated their effects on proliferation of SCC.

As shown in Fig. 5*A*, the H520 line expresses muscarinic receptors as shown by the ability of the nonselective muscarinic antagonist atropine to block the ACh-induced increase in intracellular calcium. M3-selective antagonists similarly blocked the ACh-induced increase in intracellular calcium (data not shown). The M3-selective mAChR antagonist darifenacin also blocked the proliferative effect of nicotine on H520 cells (Fig. 5*B*). This is consistent with our prior report that darifenacin blocks both basal and ACh-induced phosphorylation of MAPK (pERK1/2; ref. 22), and as shown in Fig. 6, MAPK activation represents an intersection of both nAChR and mAChR activation. Finally, as shown in Fig. 5*C* and *D*, darifenacin also significantly inhibited growth of SCC cells both *in vitro* and in nude mice. This suggests that M3-selective antagonists may have clinical utility to slow the

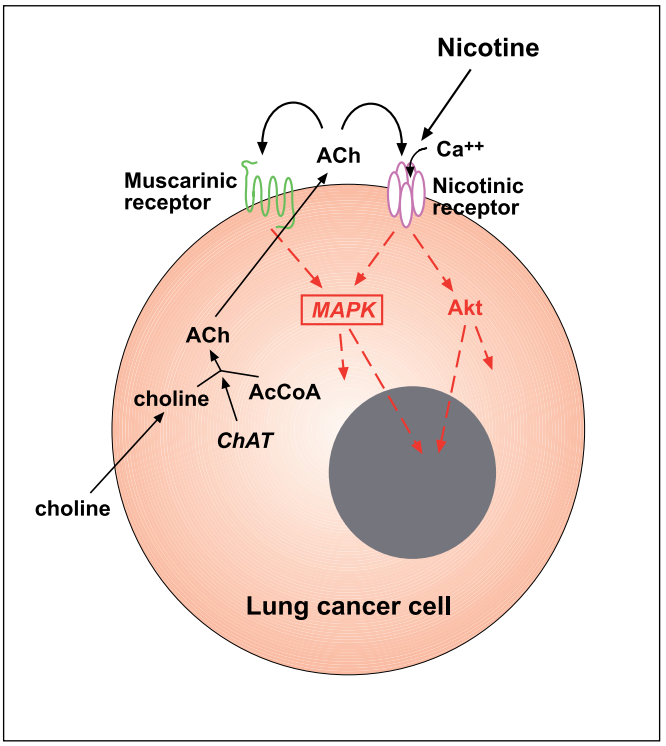

Figure 6. Model of cholinergic signaling in SCC. Exogenous nicotine activates MAPK and Akt proliferative pathways through nicotinic cholinergic receptors. Endogenous ACh similarly activates both MAPK and Akt pathways through both muscarinic and nicotinic receptors. Thus, muscarinic antagonists have potential to block proliferation induced both by exogenous nicotine and endogenous ACh as well as antagonizing the up-regulation in cholinergic signaling induced by nicotine.

growth and progression of SCC by blocking the proliferative stimuli caused by endogenous and exogenous cholinergic stimulation. Given their widespread clinical use, it may be possible to derive epidemiologic data on lung cancer survival from individuals treated long term with M3 antagonists, although such studies have yet to be reported.

Disclosure of Potential Conflicts of Interest

P. Song, H.S. Sekhon, and E.R. Spindel have submitted a provisional patent on the potential use of M3 muscarinic antagonists to treat cancer. E.R. Spindel: commercial research grants, Novartis Pharma and Boehringer-Ingleheim. The other authors disclosed no potential conflicts of interest.

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Pingfang Song, Harmanjatinder S. Sekhon, Xiao Wen Fu, et al.

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