Regulation of Integrin-Mediated Adhesion by Muscarinic Acetylcholine Receptors and Protein Kinase C in Small Cell Lung Carcinoma*

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Study objectives: Improved understanding of the phenotypic characteristics of small cell lung cancer (SCLC) cells may facilitate the development of new therapies for this bronchogenic malignancy with early metastases. Herein we investigate whether activation of the M₃ subtype of muscarinic acetylcholine receptor (mAChR) expressed on SCLC cells affects β₁-integrin-mediated adhesion of these cells.

Design: Adhesion of the SCLC cell lines SCC-9 and NCI-H345 to extracellular matrix (ECM) proteins was investigated. Cell adhesion was quantitied by labeling the cells with either toluidine blue dye and measuring optical density or [³H]-thymidine and measuring β-activity. Fluorescence-activated cell sorting was used to quantify the SCLC cell surface expression of β₁-integrins.

Setting: Experiments were conducted in the Molecular Pharmacology Laboratory, Guthrie Research Institute.

Measurements and results: Activation of mAChR with the agonist carbachol (10 μM, 1.5 h) significantly increases adhesion of the SCC-9 SCLC cell line to the ECM proteins laminin and collagen types I and IV. In contrast, mAChR activation does not alter the adhesion of SCC-9 cells to vitronectin, fibronectin, poly-L-lysine, or bovine serum albumin. Carbachol also does not alter the adhesion of NCI-H345 SCLC cells that lack functional mAChR. Preincubation of SCC-9 cells with the AIB2 blocking antibody to β₁-integrin inhibits mAChR-induced adhesion to ECM proteins. Immunofluorescence analysis indicates that mAChR activation does not alter the surface expression of β₁-integrins by SCC-9 cells. Direct stimulation of protein kinase C (PKC) by treatment with phorbol 12-myristate 13-acetate (PMA) (10 nM, 1.5 h) increases the adhesion of both the SCC-9 and NCI-H345 cell lines to ECM proteins. These results indicate that direct activation of PKC or stimulation of M₃ mAChR (which results in increased PKC activity) increases the binding activity of β₁-integrins, resulting in increased adhesion of SCLC cells to ECM proteins.

Conclusions: The ability of mAChR to regulate SCLC proliferation and adhesion suggests that activation of these receptors may be used to alter SCLC tumorigenesis and metastasis.

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Key words: integrins; metastasis; muscarinic acetylcholine receptors; protein kinase C; small cell lung carcinoma

Abbreviations: BSA=bovine serum albumin; CBS= calf bovine serum; ECM=extracellular matrix; mAChR=muscarinic acetylcholine receptor; PBS=phosphate-buffered saline solution; PKC=protein kinase C; PMA=phorbol 12-myristate 13-acetate; SCLC=small cell lung carcinoma

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Small cell lung carcinoma (SCLC) accounts for 20 to 25% of all bronchogenic malignancies and is associated with the poorest 5-year survival. The disease has a propensity for widespread metastases even in the early stages, so surgery has a limited role with few exceptions.¹⁻³ Although SCLC is initially highly responsive to combination chemotherapy, resistance develops rapidly and relapses are common.⁴ The inadequacy of current treatment regimens for SCLC underscores the need to develop new therapeutic approaches for this disease.

Characterizing the factors that regulate SCLC
metastasis will facilitate the development of new therapies. The adherence of tumor cells to each other and to extracellular matrix (ECM) proteins plays an important role in the metastasis of SCLC, as well as other types of cancer. Loss of cell-cell adhesion initiates metastasis by promoting dissociation of tumor cells from the primary tumor mass. Dynamic changes in adhesion to ECM proteins subsequently allow metastasizing cells to migrate through tissues, enter and exit the circulatory system, and ultimately invade distant organs. SCLC cells express cadherins that regulate cell-cell adhesion, and integrins that regulate adhesion to ECM proteins. Modifying the activity of cadherins and integrins expressed by SCLC cells will alter the adhesive interactions of these cells and thus may alter SCLC metastasis.

SCLC cells express a variety of heterotrimeric G protein-coupled receptors that are of potential therapeutic interest due to their ability to regulate SCLC cell proliferation. Among these receptors, the M₃ muscarinic acetylcholine receptor (mAChR) is particularly interesting because it also regulates SCLC cell-cell adhesion. We previously reported that activation of the M₃ mAChR induces E-cadherin-mediated adhesion of SCLC cells. E-cadherin-mediated adhesion induced by mAChR activation may prohibit tumor dissociation and thus diminish SCLC metastasis. This is supported by previous reports that E-cadherin-mediated adhesion suppresses the invasive behavior of tumor cells. Activation of the M₃ mAChR also inhibits the proliferation of SCLC cells. The ability of mAChR to inhibit SCLC proliferation and induce cadherin-mediated adhesion indicates that these receptors, or the signaling pathways activated by these receptors, are potentially valuable targets for diminishing SCLC tumorigenesis and metastasis.

In addition to regulating cadherin-mediated adhesion, M₃ mAChR may also regulate integrin-mediated adhesion of SCLC cells. Previous studies provide evidence that M₃ mAChR regulates the activity of integrins in other cell types. The p125 focal adhesion kinase, which plays a role in integrin-mediated adhesion, is activated by mAChR stimulation. Stimulation of M₃ mAChR also activates protein kinase C (PKC), which is known to regulate integrin activity in diverse cell types. Based on these findings, we hypothesized that activation of M₃ mAChR modulates integrin-mediated adhesion of SCLC cells.

In the present study, we determined whether mAChR activation alters the activity of β₁ integrins in SCLC cells. Expression of α₃β₁-integrin heterodimers occurs in a significant number of SCLC lines. These glycoproteins permit adhesion of SCLC cells to ECM proteins and thus contribute to SCLC invasion and migration. We report herein that mAChR activation induces β₁-integrin-mediated adhesion of SCLC cells to collagen type IV. Direct activation of PKC with phorbol esters also increases adhesion of SCLC cells to collagen type IV. This suggests that PKC activation is one of the signals that activates integrins upon stimulation of mAChR in SCLC cells.

**Materials and Methods**

**Reagents**

Poly-L-lysine (molecular weight, 30,000 to 70,000), bovine serum albumin (BSA), and purified ECM proteins (collagen types I and IV, laminin, vitronectin, and fibronectin) were purchased (Sigma Chemical Co; St Louis). Fluorescein-labeled antimouse IgG was obtained (Fisher Scientific Co; Pittsburgh). The A1B2 antibody against β₁-integrin was a generous gift from Dr. Caroline Dansky (University of California, San Francisco). The 4B4 antibody against β₁-integrin (hybridoma clone 4B4LD39LDH8) was purchased (Coulter; Marietta, Ga), and the α₂ antibody against α₂-integrin (hybridoma clone P1E9) was purchased (Telios; San Diego). A control antibody reactive with keyhole limpet hemocyanin was obtained (Becton-Dickinson; Mountain View, Calif). The HECD mouse monoclonal antibody against human E-cadherin was purified from hybridoma culture supernatants. Primary antibodies were dialyzed against RPMI-1640 media (GIBCO BRL; Gaithersburg, Md) before being used in functional assays of living cells.

**Cell Culture**

The SCLC cell line SCC-9 was established from a biopsy specimen and has been extensively characterized. The SCLC cell line NCI-H345 was obtained (American Type Culture Collection; Beltsville, Md). Both cell lines were cultured in RPMI-1640 medium, 10% heat-inactivated calf bovine serum (CBS), glutamine (0.5 mg/mL), penicillin (20 U/mL), and streptomycin sulfate (20 μg/mL). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂/95% air at densities that promoted exponential proliferation.

**Coating of Microtiter Plates With ECM Proteins**

Wells of microtiter plates were coated with poly-L-lysine (30 μg/mL phosphate-buffered saline solution [PBS]) or BSA (30 μg/mL PBS) by adding 100 μL of the protein solutions to each well. After incubation for 3 h at 25°C, the wells were washed three times with PBS (200 μL per well) and allowed to air dry. Microtiter wells were coated with collagen type I (100 μg/mL), collagen type IV (100 μg/mL PBS), laminin (30 μg/mL), vitronectin (30 μg/mL), or fibronectin (30 μg/mL) by adding 20 μL of the protein solutions to each well. After incubation for 3 h at 25°C, the wells were washed once with PBS (200 μL per well) and allowed to air dry. The microtiter plates were used within 24 h for adhesion assays.

**Adhesion Assays**

Adhesion assays were performed using two methods that produced similar results. In the first method, 50 μL of culture
medium containing 1.5×10⁷ cells were added to microtiter wells that were precoated with different proteins. The cells were incubated in the wells in the absence or presence of drugs for 90 min at 37°C. Nonadherent cells were removed by centrifuging the inverted plates (200g; 1 min). Adherent cells remaining in the wells were fixed with formalin (5% in PBS) for 18 h at 4°C, and stained with toluidine blue (1% in PBS) for 1 h at 25°C. After washing three times with PBS, the adherent cells were lysed with sodium dodecyl sulfate (1% in PBS). The optical density of the released dye was measured at 620 nm using an enzyme-linked immunosorbent assay reader (Dynamech; Oxard, Calif).

In the second method, cells were incubated with ³H-thymidine (3.0 TBq/mmol, 81 Ci/mmol) for 24 h and washed three times in RPMI containing 1% CBS. The ³H-thymidine-labeled cells were added to the precoated plates (2×10⁷ cells per 100 µL culture medium per well) and incubated in the absence or presence of drugs for 90 min at 37°C. Nonadherent cells were removed by shaking the inverted plates, followed by washing the wells two times with PBS. The remaining adherent cells were incubated for 20 min with trypsin/EDTA solution (0.05% trypsin, 0.53 mM EDTA) to lift them from the plates, and collected on filters using an automatic cell harvester (Skatron; Sterling, Va). Radioactivity on the filters was counted with a β-counter (LS-7000; Beckman Instruments; Fullerton, Calif).

Immunofluorescence Assays

Cells were incubated (30 min, 4°C) with the 4B4 antibody to β₁-integrin diluted in PBS containing 1% BSA, washed twice in ice-cold PBS/1% BSA, and incubated (30 min, 4°C) with a 1:100 dilution of fluorescein-labeled antibody to mouse IgG. After washing, the cells were fixed in paraformaldehyde (1% in PBS) and analyzed using a fluorescence-activated cell sorter (Becton-Dickinson Immunocytometry Systems; Mountain View, Calif). Nonspecific, background immunofluorescence was determined by incubating the cells with control antibody reactive with keyhole limpet hemocyanin (Becton-Dickinson) that is not expressed by human cells, followed by fluorescein-labeled secondary antibody. The results are presented as the mean fluorescence intensity of the cell population.

Statistical Analysis

Statistical analysis of the data was performed using Student’s t distribution with a confidence limit of 95%.

RESULTS

The ability of the SCC-9 and NCI-H345 SCLC cell lines to adhere to different ECM proteins was compared with their adherence to poly-L-lysine and BSA. Adhesion to ECM proteins is mediated by β₁-integrin heterodimers. In contrast, adhesion to poly-L-lysine is not mediated by integrins, but occurs due to charge interactions, while BSA should not support adhesion. As expected, both cell lines maximally adhere to poly-L-lysine and minimally adhere to BSA (Fig 1). Adhesion of both cell lines to collagen type IV and laminin is significantly greater than adhesion to BSA (Fig 1). Additionally, the NCI-H345 cell line exhibits significant adhesion to collagen type I (Fig 1, bottom [B]). The adhesion of NCI-H345 and SCC-9 cells to collagen and laminin indicates that these cells express functional β₁-integrin heterodimers, in agreement with previous reports.⁷,⁹

The effects of mAChR activation on integrin-mediated adhesion of SCLC cells were tested by exposing the cells to the mAChR agonist carbachol. Although SCC-9 cells express functional mAChR,⁷,¹² NCI-H345 cells do not express functional mAChR and therefore should not respond to carbachol.⁷ As expected, carbachol does not alter the adhesion of NCI-H345 cells (Fig 1, top [A]). In contrast, carbachol significantly increases the adhesion of SCC-9 cells to collagen types I and IV and laminin (Fig 1, top [A]). This finding provides evidence that mAChR activation stimulates integrin activity in SCC-9 cells.
The ability of mAChR to regulate integrin activity was further characterized by examining the carbachol-mediated adhesion of SCC-9 cells to collagen type IV. Concentrations of carbachol that stimulate adhesion of SCC-9 cells to collagen type IV (Fig 2) are similar to the concentrations of carbachol that inhibit cell proliferation\(^{15}\) and induce E-cadherin-mediated cell-cell adhesion\(^2\) of SCC-9 cells. The mAChR antagonist atropine inhibits carbachol-induced adhesion of SCC-9 cells to collagen type IV (Fig 3), indicating that this response is mediated by mAChR activation.

The involvement of \(\beta_1\)-integrins in the mAChR-induced adhesion of SCC-9 cells was tested by exposing the cells to the AIIB2 antibody. The AIIB2 antibody blocks \(\beta_1\)-integrin and inhibits the \(\beta_1\)-integrin-mediated adhesion of a variety of cell types.\(^{25}\) Incubation of SCC-9 cells with the AIIB2 antibody inhibits the carbachol-induced adhesion of the cells to collagen type IV, but does not alter the adhesion of the cells to poly-L-lysine (Fig 4, top [A]). As a control experiment, we also tested the effects of the \(\alpha_2\)-antibody on the carbachol-induced adhesion of SCC-9 cells. The \(\alpha_2\)-antibody blocks \(\alpha_2\)-integrins, which are underexpressed by SCC-9 cells.\(^7\) As expected, the \(\alpha_2\)-antibody does not alter the carbachol-induced adhesion of SCC-9 cells (Fig 4, bottom [B]).

The demonstration that the AIIB2 antibody directed against \(\beta_1\)-integrin specifically abrogates the mAChR-induced adhesion of SCC-9 cells to collagen type IV indicates that \(\beta_1\)-integrins participate in this adhesive event.

Stimulation of \(M_3\) mAChR may increase the expression or activity of \(\beta_1\)-integrins. We used fluorescence-activated cell sorting to quantitate the surface expression of \(\beta_1\)-integrin on SCC-9 cells. Immunofluorescent staining by the 4B4 antibody to \(\beta_1\)-integrin is similar in SCC-9 cells that were untreated or preincubated with carbachol to activate mAChR (Fig 5). This indicates that integrin-mediated adhesion induced by mAChR stimulation is due to activation of \(\beta_1\)-integrin, rather than increased expression of \(\beta_1\)-integrin.

Stimulation of \(M_3\) mAChR elicits a variety of intracellular signals, including PKC activation.\(^{17}\) Activation of PKC may stimulate integrin-mediated adhesion of SCC-9 cells, as it does in other cell types.\(^{18-24}\) In support of this hypothesis, we found that direct activation of PKC with phorbol 12-myristate 13-acetate (PMA) stimulates adhesion of SCC-9 cells to collagen type IV (Fig 6, left [A]). This finding suggests that PKC activation is one of the signals that induce integrin-mediated adhesion of SCC-9 cells following mAChR stimulation. Activations

![Figure 2](image-url) - Increasing concentrations of carbachol stimulate adhesion of SCC-9 cells to collagen type IV. SCC-9 cells were allowed to adhere for 90 min to poly-L-lysine, collagen type IV, or BSA in the presence of increasing concentrations of carbachol. Adhesion was quantitated as described in the legend to Figure 1. Results are the means±1 SEM from at least three independent experiments. The adhesion of cells to collagen type IV is significantly different from adhesion to poly-L-lysine or to BSA in the presence of \(<10^{-4}\) M carbachol (p<0.05). The cells adhere equally well to collagen type IV and poly-L-lysine in the presence of \(10^{-4}\) M or \(10^{-5}\) M carbachol.
tion of PKC with PMA also stimulates adhesion of NCI-H345 cells to collagen type IV (Fig 6, left [A] and center [B]). This finding indicates that adhesion induced by PKC activation is not restricted to SCC-9 cells, but also occurs in other SCLC cell lines.

Activation of PKC induces greater adhesion of SCC-9 cells than NCI-H345 cells to collagen type IV (Fig 6, left [A] and center [B]). E-cadherin-mediated cell-cell adhesion induced by PKC activation may contribute to the increased adhesive response of SCC-9 cells. We previously reported that mAChR or PKC activation increases E-cadherin-mediated adhesion of SCC-9 cells, but not NCI-H345 cells. E-cadherin-mediated adhesion increases the size of SCC-9 cell aggregates, which would result in an increased number of SCC-9 cells remaining in the collagen-coated plates after exposure to carbachol or PMA. The participation of E-cadherin in the adhesive response of SCC-9 cells was tested by exposing the cells to the HECD antibody, which abolishes E-cadherin-mediated adhesion. We found that mAChR or PKC activation significantly increases the adhesion of SCC-9 cells to collagen type IV when E-cadherin-mediated adhesion is abrogated by the HECD antibody (Fig 6, right [C]). However, inhibiting cell-cell adhesion with the HECD antibody diminishes the size of the SCC-9 cell aggregates adhering to collagen type IV, resulting in an adhesive response that more closely resembles the response of NCI-H345 cells (Fig 6).

**DISCUSSION**

This study demonstrates that PKC or mAChR activation increases integrin-mediated adhesion of SCLC cells. Activation of mAChR increases the adhesion of SCC-9 cells to collagen type IV. This adhesive event is mediated by βι-integrins, because it is abrogated by the AIIB2 antibody that blocks βι-integrin function. Our results further suggest that PKC activation is one of the signals that induce integrin-mediated adhesion upon stimulation of mAChR.

The adhesion of SCLC cells to ECM proteins depends on the expression of βι-integrin heterodimers. Various types of βι-integrin heterodimers are expressed by different SCLC cell lines, affecting the ability of these cell lines to adhere to specific ECM proteins. Previous reports indicate that SCLC cells bind to laminin using βι-integrins. Our results indicate that SCLC cells also adhere to collagen type IV using βι-integrins.

The ability of SCLC cells to adhere to ECM proteins depends on the activation state of the integrins expressed by the cells. We found that
SCC-9 cells normally adhere poorly to collagen type IV, but exhibit increased adhesion to collagen type IV following mAChR stimulation. Stimulation of mAChR does not alter surface expression of \( \beta_1 \)-integrin by SCC-9 cells. This indicates that integrin-mediated adhesion induced by mAChR stimulation is due to activation of \( \beta_1 \)-integrins, rather than increased \( \beta_1 \)-integrin surface expression.

\( \beta_1 \)-integrins are activated by a variety of extracellular and intracellular signals, including PKC activation.\(^{18-24} \) Activation of PKC following mAChR stimulation may be the signal that increases \( \beta_1 \)-integrin activity in SCLC cells. Consistent with this, we found that activation of PKC with PMA increases the adhesion of SCC-9 cells and NCI-H345 cells to collagen type IV. Several recent studies provide evidence that PKC stimulation activates integrins by increasing the lateral mobility of integrins in the plasma membrane.\(^{23,24} \) Similar events may occur following mAChR or PKC activation in SCLC cells. The expression of specific PKC isoenzymes\(^{26} \) or abnormally active forms of PKC\(^{27} \) by different SCLC cell lines may also affect integrin activity in SCLC cells.

Increased integrin activity may have profound effects on SCLC tumors due to altered interactions of the tumor cells with the surrounding connective tissue. Cell proliferation, differentiation, and apopto-

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**Figure 4.** \( \beta_1 \)-integrins are required for mAChR-induced adhesion of SCC-9 cells to collagen type IV. SCC-9 cells were preincubated for 15 min in the absence or presence of the AIIβ2 antibody to \( \beta_1 \) integrin (top, A) or the α2-antibody to α2-integrin (bottom, B). The cells were allowed to adhere for 90 min to poly-L-lysine, collagen type IV, or BSA in the absence or presence of 10 \( \mu \)M carbachol. Adhesion was measured as indicated in the legend to Figure 1. Results are the means±1 SEM from at least three independent experiments. Asterisks indicate values from carbachol-treated cells that are significantly different compared with untreated cells adhering to the same protein (**: \( p<0.01 \); *: \( p<0.05 \)).

**Figure 5.** Activation of mAChR does not alter surface expression of \( \beta_1 \)-integrin by SCC-9 cells. SCC-9 cells were incubated in suspension for 60 min in the absence or presence of 10 \( \mu \)M carbachol, and then immunofluorescently stained for \( \beta_1 \)-integrin surface expression. Results shown are the means±1 SEM of four determinations from two independent experiments.

**Figure 6.** Activation of PKC induces adhesion of SCC-9 and NCI-H345 cells to collagen type IV. SCC-9 cells (left [A], right [C]) or NCI-H345 cells (center [B]) were prelabeled with \( ^{3} \)H-thymidine and allowed to adhere for 90 min to collagen type IV in the absence (hatched bars) or presence of 10 \( \mu \)M carbachol (solid bars) or 10 nM PMA (open bars). Nonadherent cells were removed by shaking the inverted plates, and radioactivity of the remaining adherent cells was measured. SCC-9 cells treated with the HECB blocking antibody to E-cadherin (C) were incubated with the HECB antibody for the last 30 min of the assay before removal of the nonadherent cells. This treatment with the HECB antibody was previously shown to completely inhibit E-cadherin-mediated cell-cell adhesion of SCC-9 cells. Results are the means±1 SEM from at least three independent experiments. Asterisks indicate values from carbachol- or PMA-treated cells that are significantly different compared with untreated cells (**: \( p<0.01 \); *: \( p<0.05 \)).
sis can all be influenced by the integrin-mediated adhesion of cells to specific ECM proteins found in connective tissue.28-30 Previous studies demonstrated that interaction with laminin alters the expression of neuroendocrine characteristics by SCLC cells31 and affects SCLC tumor growth.32 Activation of β1-integrins and increased adhesion to collagen type IV and laminin following mAChR or PKC stimulation may alter these or additional parameters in SCLC cells.

Changes in integrin activity will modulate SCLC metastasis by altering the ability of the cells to adhere to ECM proteins. The ECM proteins found in basal lamina may have a particularly important role in SCLC metastasis. Basal lamina consists of thin layers of specialized ECM that underlie epithelial structures. Laminin and collagen type IV are two of the ECM proteins found in basal lamina.33 Since the basal lamina must be invaded for SCLC cells to metastasize, integrin-mediated adhesion of SCLC cells to the basal lamina may affect SCLC metastasis.

Changes in integrin-mediated adhesion may also alter SCLC metastasis by affecting the migration of metastasizing SCLC cells. The integrin-mediated attachment of cells to ECM proteins is required for SCLC cell migration in vitro.32 However, the effects of altering integrin activity on the migration of metastasizing SCLC cells in vivo have not been completely characterized. Abnormal increases in integrin activity may slow migration by delaying the release of tumor cells from the ECM, or alternatively accelerate migration by promoting attachment and forward movement of tumor cells on the ECM.

Successful treatment of SCLC depends on the development of multidimensional therapeutic approaches.34 Treatment strategies that disrupt SCLC tumorigenesis and metastasis at multiple points have much greater therapeutic potential than treatments that affect only one aspect of the disease process. Activation of mAChR or PKC affects multiple aspects of SCLC biology. In addition to inducing integrin-mediated adhesion, stimulation of M3 mAChR inhibits proliferation15 and induces cadherin-mediated adhesion7 of SCLC cells. Direct activation of PKC with phorbol esters can also induce both cadherin-7 and integrin-mediated adhesion of SCLC cells, as well as altering other responses of SCLC cells.35,36 These findings indicate that mAChR and PKC warrant further investigation as targets for developing effective therapeutic strategies for SCLC.

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