

Regulation of E-cadherin-mediated Adhesion by Muscarinic Acetylcholine Receptors in Small Cell Lung Carcinoma

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Abstract. We present the first evidence that adhesion mediated by a member of the cadherin gene family can be regulated by a G protein-coupled receptor. We show that activating the M₃ muscarinic acetylcholine receptor (mAChR) rapidly induces E-cadherin-mediated adhesion in a small cell lung carcinoma (SCLC) cell line. This response is inhibited by E-cadherin antibodies, and does not occur in another SCLC cell line which expresses functional mAChR but reduced levels of E-cadherin. Protein kinase C may be involved,

since phorbol 12-myristate 13-acetate also induces E-cadherin-mediated aggregation. Immunofluorescence analyses indicate that mAChR activation does not grossly alter E-cadherin surface expression or localization at areas of cell-cell contact, suggesting mAChR activation may increase E-cadherin binding activity. Our findings suggest that G protein-coupled receptors may regulate processes involving cadherin-mediated adhesion, such as embryonic development, neurogenesis, and cancer metastasis.

CELL adhesion molecules participate in a variety of biological processes. Because altered expression or activity of these molecules may disrupt normal cell functioning, determining how adhesion is regulated is important for understanding both normal and pathological cellular events. Neoplastic transformation is one of the most well studied pathological processes which may result in part from altered cell adhesion. Anchorage-independent growth and continued proliferation in the presence of cell-cell contact (i.e., loss of contact inhibition) are two characteristics of transformation which may arise from altered expression or activity of cell adhesion molecules. Consistent with this, several neoplasms express altered levels or different types of adhesion molecules compared to their normal tissue counterparts (for review see Edelman and Crossin, 1991). In addition to participating in transformation, adhesion molecules may control invasion and metastasis of transformed cells (Behrens et al., 1989; Andersson et al., 1991; Johnson, 1991; Chen and Obrink, 1991; Frixen et al., 1991; Vleminckx et al., 1991; Zhu et al., 1991). Thus, the identification of factors which modulate cell adhesion may provide novel therapeutic approaches for tumorigenesis and metastatic disease.

Factors regulating the activity of some adhesion molecules have been characterized. For example, the binding of cell surface receptors by cytokines can increase the activity or expression of the intercellular adhesion molecule-1 (ICAM-

1)¹ (Valent et al., 1991), the neural cell adhesion molecule (NCAM) (Roubin et al., 1990), the endothelial-leukocyte adhesion molecule-1 (ELAM-1) (Montgomery et al., 1991), and members of the integrin family (for review see Hynes, 1992). In contrast, no cytokine has yet been identified which regulates adhesion mediated by members of the cadherin family. Cadherins play an important role in embryonic compaction, morphogenesis, and neurogenesis (for review see Takeichi, 1991), yet the factors which regulate cadherin-mediated adhesion have not been determined. The ability of cadherins to inhibit invasion and metastasis of transformed cells (Behrens et al., 1989; Chen and Obrink, 1991; Frixen et al., 1991; Vleminckx et al., 1991) provides further motivation to elucidate the regulation of cadherin-mediated adhesion.

Activation of protein kinase C by phorbol esters increases adhesion mediated by some molecules, such as E-cadherin (Winkel et al., 1990), ICAM-1 (Patarroyo et al., 1987), ELAM-1 (Montgomery et al., 1991), and members of the integrin family (Symington et al., 1989; Wilkins et al., 1991; Hibbs et al., 1991a; Valmu et al., 1991). This suggests that stimulation of cell surface receptors which are functionally coupled to protein kinase C may alter adhesion molecule activity. This possibility is supported by recent findings that cells transfected with receptors which functionally couple to

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1. Abbreviations used in this paper: mAChR, muscarinic acetylcholine receptor; CEA, carcinoembryonic antigen; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methiodide; ELAM-1, endothelial-leukocyte adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; NCAM, neural cell adhesion molecule; SCLC, small cell lung carcinoma.

protein kinase C exhibit anchorage-independent growth and loss of contact inhibition (Young et al., 1986; Julius et al., 1989, 1990; Gutkind et al., 1991). This phenotype is induced by transfected muscarinic acetylcholine receptors (mAChR) only in the presence of agonist, indicating that second messengers generated by the receptors regulate this response (Gutkind et al., 1991). Protein kinase C may be one of the required effectors, since transformation is induced only by the mAChR subtypes M₁, M₃, and M₅, which activate protein kinase C, and not by the mAChR subtypes M₂ and M₄ which activate other second messenger pathways (Gutkind et al., 1991).

We are investigating factors controlling the adhesion of small cell lung carcinoma (SCLC) cells. SCLC cells express NCAM (Kibbelaar et al., 1989; Patel et al., 1989; Aletsee-Utrecht et al., 1990; Rygaard et al., 1992), ICAM-1 (Boyd et al., 1989), carcinoembryonic antigen (CEA) (Goslin et al., 1983; Bepler et al., 1989), and members of the integrin (Hemler et al., 1990; Feldman et al., 1991) and cadherin (Shimoyama et al., 1989; Rygaard et al., 1992) gene families. SCLC cells also express receptors for neurotransmitters (Sorenson et al., 1983; Cunningham et al., 1985; Maneckjee and Minna, 1990; Williams and Lennon, 1990) and autocrine growth factors (for review see Weynants et al., 1990), and it is possible that activation of these receptors may modulate the expression or activity of SCLC adhesion molecules. To investigate this, we have examined the ability of the M₃ subtype of mAChR expressed by SCLC cells to regulate cell-cell adhesion. We previously reported that mAChR activation inhibits voltage-gated Ca²⁺ channel activity (Williams and Lennon, 1990) and causes cell cycle arrest (Williams and Lennon, 1991) in the SCLC cell line SCC-9. We now report that cell-cell adhesion increases upon mAChR

activation in SCC-9 cells. We have identified E-cadherin (also known as uvomorulin) as the adhesion molecule mediating this response. Our data indicate that the binding activity of E-cadherin is increased by mAChR activation, and that protein kinase C may participate in this response.

Our finding that mAChR activation increases E-cadherin-mediated adhesion provides the first evidence that E-cadherin binding activity can be regulated by activating a heterologous, cell surface receptor. Additionally, this is the first report that activation of a neurotransmitter receptor can increase the binding activity of a cell adhesion molecule. These findings may have important implications for our understanding of the factors which regulate embryonic compaction, morphogenesis, and neurogenesis, all of which involve cadherin-mediated adhesion. These findings also support the hypothesis that neoplastic transformation induced by mAChR or other G protein-coupled receptors may involve receptor-mediated changes in adhesion molecule activity. Additionally, our data suggest a novel mechanism to induce E-cadherin-mediated adhesion, and thus possibly alter metastasis and invasion of carcinomas expressing this adhesion molecule.

Materials and Methods

Reagents

The primary antibodies used in this study are described in Table I. The disparate concentrations of the primary antibodies provided by the suppliers prohibited using equal concentrations of the different antibodies in the assays. As an alternative, an attempt was made to use maximal concentrations of antibodies. To do this, all antibodies used in a particular assay were diluted by the same factor. Primary antibodies were dialyzed against RPMI 1640 media (GIBCO BRL, Gaithersburg, MD) before being used in functional assays of living cells. FITC-labeled secondary antibodies (Fisher

Table I. Primary Antibodies Used in the Assays

Name used in text	Antigenic specificity	Hybridoma clone	Isotype*	Stock concentration (μg/ml)	Supplier
α-2	α ₂ -integrin	P1E6	IgG ₁	NA‡	Telios
α-L	α _L -integrin	MHM24	IgG ₁	114	Dako
α-M	α _M -integrin	2LPM19C	IgG ₁	82	Dako
β-1	β ₁ -integrin	4B4LDC9LDH8	IgG ₁	1,000	Coulter
β-2	β ₂ -integrin	MHM23	IgG ₁	206	Dako
Control	Keyhole Limpet hemocyanin	X40	IgG ₁	50	Becton-Dickinson
CD57	CD57	HNK-1	IgM	12	Becton-Dickinson
CEA	CEA	A5B7	IgG ₁	0.04	Dako
DECMA	E-cadherin	DECMA-1	rat IgG ₁	9,600	Sigma
HECD	E-cadherin	HECD-1	IgG ₁	500	Dr. S. Thacher
Leu54	ICAM-1	LB-2	IgG _{2b}	3	Dr. M. Takeichi
N-cad	N-cadherin	GC-4	IgG ₁	2,800	Becton-Dickinson
NCAM	NCAM	MY31	IgG ₁	50	Sigma
P1B5	α ₃ -integrin	P1B5	IgG ₁	NA‡	Becton-Dickinson
WEHI	ICAM-1	WEHI-CAM-1	IgG _{2b}	1,000	Dr. J. Fecondo
					Dr. A. Boyd

* All antibodies are from the mouse, except where noted.

‡ Not available. IgG concentrations are not available from the supplier. Lots used were 1A0384 (hybridoma clone P1E6) and 1A0453 (hybridoma clone P1B5).

Scientific Co., Pittsburgh, PA) included goat anti-mouse IgG, goat anti-mouse IgM, and goat anti-rat IgG. PMA and the inactive drug 4- α -phorbol (Sigma Chem. Co., St. Louis, MO) were dissolved in anhydrous dimethylsulfoxide (Sigma Chem. Co.) at a stock concentration of 6 mM, and diluted in culture medium for assays. Carbachol, atropine, pertussis toxin (Sigma Chem. Co.), and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) (Research Biochems. Inc., Natick, MA) were diluted directly in culture medium. [35 S]methionine in the form of Tran 35 S-label was purchased from ICN Biomedicals Inc. (Irvine, CA). Rat anti-mouse kappa light chain coupled to Sepharose 4B was purchased from Zymed Labs., Inc. (San Francisco, CA). Other reagents were obtained from sources listed in the text.

Cell Culture

The SCLC cell line SCC-9 line was established from a biopsy specimen of SCLC which had metastasized to the skin. Extensive characterization of this cell line (Cunningham et al., 1985; Williams and Lennon, 1990, 1991) indicates it is clonal and expresses mAChR of M₃ subtype, but does not express nicotinic acetylcholine receptors of the type found in skeletal muscle (Cunningham et al., 1985). The SCLC cell lines NCI-H146, NCI-H345, NCI-H209, NCI-H69, and NCI-H82 were obtained from the Amer. Type Culture Collection (Rockville, MD). All SCLC cell lines used in this study grow as floating aggregates in tissue culture. The SA-4B cell line is a clonal line of human B cells transformed with Epstein-Barr virus, as previously described (Williams et al., 1992). All cell lines were cultured in RPMI 1640 medium containing 10% calf bovine serum (Hyclone Labs., Logan UT), glutamine (0.3 mg/ml), penicillin (20 U/ml), and streptomycin sulfate (20 μ g/ml), at 37°C in a humidified atmosphere of 5% CO₂/95% air. Cultures were maintained at cell densities which promoted exponential growth.

Measurement of Intracellular Free Ca²⁺ Concentration

Cells were incubated in culture medium containing Indo-1 AM (4 μ M) and pluronic (0.1%) (Molecular Probes, Inc., Eugene, OR) for 30 min at 37°C, diluted 1:1 with culture medium, incubated another 30 min, and washed. The ratio of intracellular Indo-1 fluorescence at 390 and 500 nm was measured with a Facstar-Plus (Becton-Dickinson Immunocytometry Sys., Mountain View, CA). Ratios were converted to Ca²⁺ concentrations as previously described (Williams and Lennon, 1990).

Cell-Cell Adhesion Assays

Cells were incubated in culture medium with or without antibodies (1:5 dilution, 30 min, 37°C), and then gently shaken with or without drugs (1 h, 37°C). Cells were transferred to microscope slides coated with poly-L-lysine, fixed in Trump's solution (1% glutaraldehyde and 4% formaldehyde in sodium phosphate buffer), and mounted in PBS containing glycerol (1%). The mean area of cell aggregates in eight microscope fields in each sample was determined by computer-assisted morphometry using an IBAS Image Analysis System (Carl Zeiss Inc., Thornwood, NY).

Immunofluorescence Assays

For quantitative analysis of surface proteins, cells were incubated (30 min, 4°C) with primary antibody diluted 1:5 in PBS containing 1% BSA, washed twice in cold PBS/1% BSA, and incubated (30 min, 4°C) with a 1:100 dilution of fluorescein-labeled secondary antibody specific for the primary antibody. After washing, the cells were fixed in paraformaldehyde (1% in PBS) and analyzed using a fluorescence-activated cell sorter (Becton-Dickinson Immunocytometry Sys.). When primary antibodies specific for cadherins were used, PBS was replaced with Krebs buffer (118 mM NaCl, 4.7 mM KCl, 2 mM CaCl₂, 1.2 mM MgCl₂, 24.9 mM NaHCO₃, pH 7.4). Nonspecific, background immunofluorescence was determined by incubating cells with control antibody reactive with keyhole limpet hemocyanin, which 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, and as the percent of cells with fluorescence intensity greater than cells incubated with control antibody.

For photomicroscopy, cells were incubated in culture medium in the presence or absence of drugs (1 h, 37°C), and then centrifuged onto coverslips coated with poly-L-lysine. Cells were fixed and permeabilized in formalin (3%, 30 min, 4°C) and Triton-X 100 (0.1%, 5 min, 22°C), washed three times in Krebs buffer, and incubated in Krebs buffer containing 1% BSA (30 min, 22°C). After incubation with HECD or control antibody diluted 1:100 in Krebs buffer/1% BSA (1 h, 22°C), the cells were washed

three times and incubated with a 1:100 dilution of fluorescein-labeled anti-mouse IgG (1 h, 22°C). After washing, cells were mounted in PBS containing glycerol (1%) and *p*-phenylenediamine (0.1%) and examined with a fluorescence microscope (Carl Zeiss, Inc.)

Immunoprecipitation

Cells were cultured in methionine-free RPMI 1640 medium containing calf bovine serum (10%) and [35 S]methionine (50 μ Ci/ml) for 16 h at 37°C, and lysed in lysis buffer A (1% Triton-X 100 in PBS containing 2 mM CaCl₂, pH 7.4) or lysis buffer B (0.5% sodium deoxycholate, 2% NP-40, 0.2% SDS, 1 mM Na₃VO₄, 50 mM NaCl, 25 mM Tris-HCl, pH 7.4) in the presence of protease inhibitors (50 μ M PMSF, 0.1 μ g/ml Pepstatin A, and 1 KIU/ml Aprotinin). After preclearing the lysates by incubation with rat anti-mouse kappa light chain coupled to Sepharose 4B (1 h, 4°C), supernates containing 10⁸ cpm were incubated (1 h, 4°C) with HECD or control antibody diluted 1:10 in dilution buffer (10 mM Tris-HCl, 140 mM NaCl, 2 mM CaCl₂, 0.05% Triton-X 100, 0.1% BSA, pH 8.0). The samples were shaken with anti-mouse kappa light chain coupled to Sepharose 4B (1 h, 4°C) and sequentially washed in dilution buffer, Tris-Saline (10 mM Tris-HCl, 140 mM NaCl, 2 mM CaCl₂, pH 8.0), and 50 mM Tris-HCl (pH 6.8). Immunoprecipitates were boiled in sample buffer (50 mM Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, pH 6.8), centrifuged, and the resulting supernates were subjected to PAGE (5% stacking gel, 10% separating gel) as previously described (Williams and Lennon, 1992). Dried gels were subjected to autoradiography.

Results

Expression of Functional mAChR by SCLC Cells

We previously demonstrated that SCC-9 cells express mAChR of the M₃ subtype (Williams and Lennon, 1990). Functional activity of these receptors is indicated by a rise in intracellular free Ca²⁺ caused by the agonist carbachol, and a loss of this response caused by 4-DAMP, an antagonist specific for the M₃ subtype of mAChR (Fig. 1). We found that NCI-H146 cells also exhibit changes in intracellular free Ca²⁺ concentrations in response to carbachol and 4-DAMP, indicating the presence of functional mAChR on these cells (Fig. 1). The slightly different Ca²⁺ responses of SCC-9 and NCI-H146 cells to carbachol (Fig. 1) may reflect quantitative differences in mAChR expression or variations in Ca²⁺ mobilization between these two cell lines. Further evidence that NCI-H146 cells express functional mAChR is the ability of carbachol to inhibit DNA synthesis in these cells (data not shown), similar to the effects of mAChR activation in SCC-9 cells (Williams and Lennon, 1991). In contrast to other SCLC cell lines, NCI-H345 cells do not exhibit changes in intracellular free Ca²⁺ concentration (Fig. 1) or DNA synthesis (data not shown) when exposed to mAChR agonists, indicating NCI-H345 cells do not express functional mAChR.

SCLC Cell-Cell Adhesion Induced by mAChR Activation or PMA Treatment

We observed that cell-cell adhesion and compaction of SCC-9 cells increases dramatically within 15 min of exposure to carbachol, and remains high during the first 4 h of treatment (Fig. 2 C). Thereafter, cell-cell adhesion declines, reaching normal levels after 12 h of continuous carbachol exposure. Using computer-assisted morphometry, we found that maximal concentrations of carbachol increase the size of SCC-9 aggregates by 200–300% (Fig. 3). This is due to mAChR activation because atropine or 4-DAMP abrogates the effects of carbachol (data not shown). Surprisingly, cell-cell adhesion of NCI-H146 cells is not affected by carbachol (Fig. 2 D),

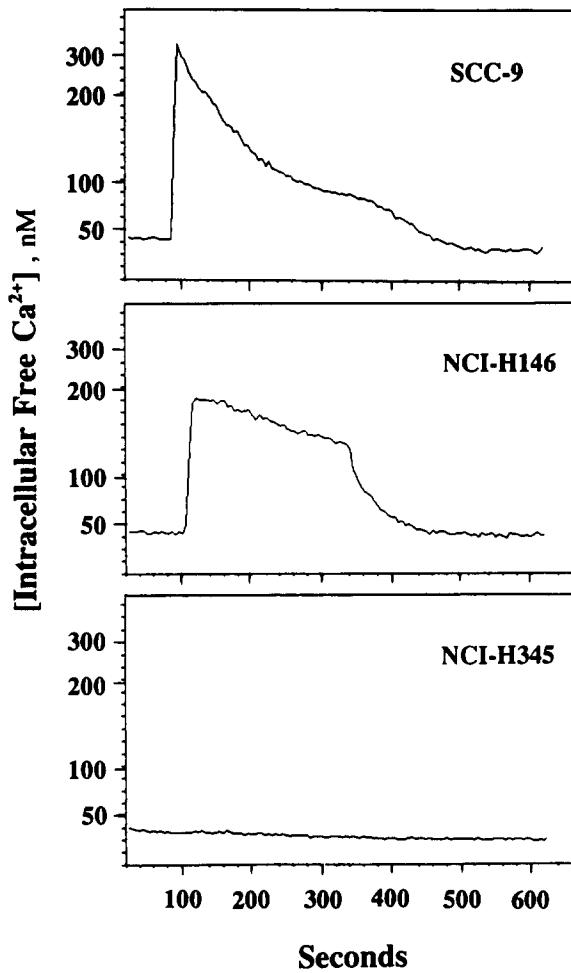


Figure 1. Functional mAChR are expressed by SCC-9 and NCI-H146 cells. In SCC-9 and NCI-H146 cells, the increase in intracellular free Ca^{2+} caused by carbachol ($100 \mu\text{M}$ applied at 80 s) is inhibited by 4-DAMP ($1 \mu\text{M}$ applied at 360 s), indicating functional mAChR in these cells. NCI-H345 cells do not respond to carbachol or 4-DAMP, indicating the absence of functional mAChR. Results shown are representative of four experiments.

despite the presence of functional mAChR on these cells. Carbachol also does not alter adhesion of NCI-H345 cells.

One consequence of M_3 mAChR stimulation is activation of protein kinase C (for review see Bonner, 1989). If protein kinase C participates in the mAChR-mediated increase in SCLC adhesion, other agents which activate protein kinase C, such as PMA, should also increase SCLC adhesion. We found that PMA increases cell-cell adhesion and compaction of SCC-9 cells (Fig. 2 G), but does not alter adhesion of NCI-H146 cells (Fig. 2 H) or NCI-H345 cells. The specificity of PMA action was confirmed by the lack of effect by 4α -phorbol (Fig. 2 E). Like carbachol, PMA induces a rapid but transitory compaction of SCC-9 cells. Compaction occurs within 15 min of exposure to PMA, but is not evident after 12 h of continuous PMA exposure.

To investigate the role of pertussis toxin-sensitive G proteins in these results, we measured the effects of pertussis toxin on aggregation of SCC-9 cells. We found that pertussis toxin ($1-800 \text{ nM}$, 30-min preincubation) did not affect normal aggregation of SCC-9 cells, nor did it alter aggregation

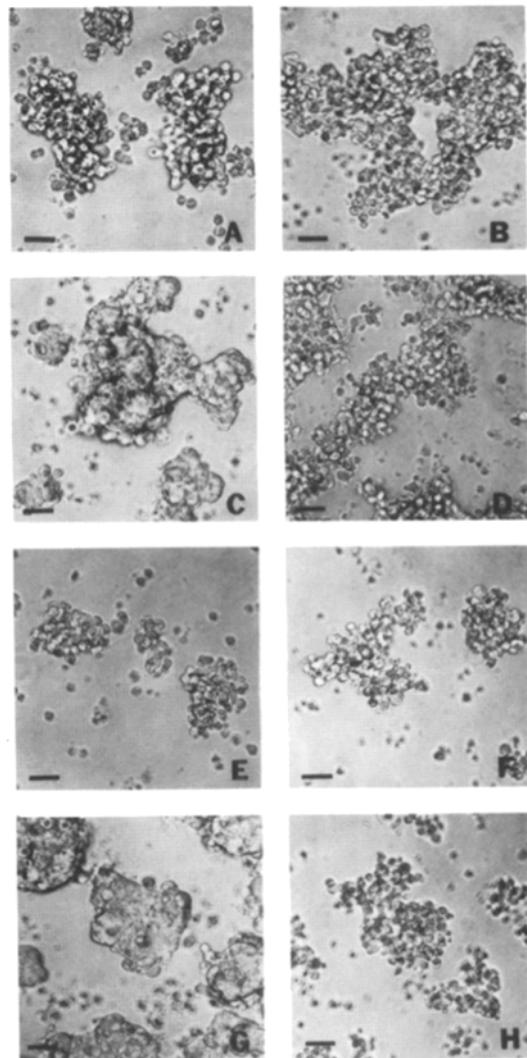


Figure 2. Compaction of SCC-9 cells is induced by mAChR activation or PMA treatment. The effects of different drugs on SCC-9 cells (A, C, E, and G) and NCI-H146 cells (B, D, F, and H) are shown. In the absence of drugs (A and B), the cells are loosely aggregated. Carbachol ($100 \mu\text{M}$, 1 h) induces compaction of SCC-9 cells (C) but not NCI-H146 cells (D). The inactive drug 4α -phorbol (60 nM , 1 h) does not affect the cells (E and F), whereas PMA (60 nM , 1 h) induces compaction of SCC-9 cells (G) but not NCI-H146 cells (H). Results shown are representative of four experiments. Bars, $50 \mu\text{m}$.

and compaction of SCC-9 cells treated with carbachol ($100 \mu\text{M}$, 1 h) or PMA (60 nM , 1 h).

Identification of the Adhesion Molecule Mediating Drug-induced Aggregation of SCC-9 Cells

We hypothesized that SCC-9 cells aggregate in response to carbachol or PMA due to increased activity or expression of a cell adhesion molecule. Low expression of this adhesion molecule by NCI-H146 cells may prohibit aggregation in response to the drugs. This hypothesis predicts that the adhesion molecule which mediates drug-induced aggregation is expressed more by SCC-9 cells than NCI-H146 cells. To investigate this, we used immunofluorescence techniques to compare the surface expression of adhesion molecules by

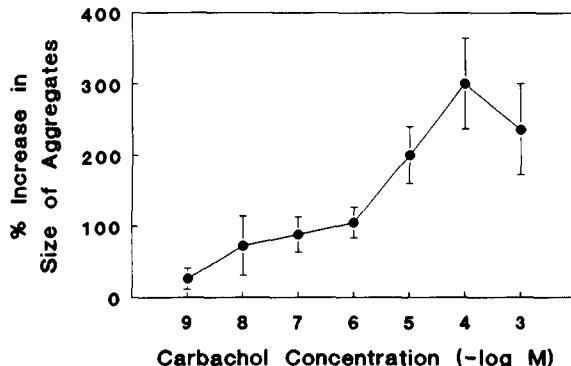


Figure 3. Aggregation of SCC-9 cells is induced by mAChR activation. Exposure to carbachol for 1 h increases the size of SCC-9 aggregates. Results shown are the mean (± 1 SEM) of eight determinations from four independent experiments.

SCC-9 and NCI-H146 cells. Several adhesion molecules are expressed similarly by SCC-9 and NCI-H146 cells, or to a lesser extent by SCC-9 cells (Fig. 4), suggesting these adhesion molecules do not mediate drug-induced aggregation. These include α_2 -integrin, β_1 -integrin, NCAM, the CD57 epitope (which may be present on NCAM), CEA, and N-cadherin (Fig. 4). There is no detectable expression of α_m -integrin, α_1 -integrin, or β_2 -integrin by either SCC-9 or NCI-H146 cells (Fig. 4).

We identified several adhesion molecules which may mediate aggregation, since they are expressed more by SCC-9 cells than NCI-H146 cells (Fig. 5). Antibodies to ICAM-1 (Leu54 and WEHI), E-cadherin (HECD and DECMA), and α_3 -integrin (PIB5) immunofluorescently stain SCC-9 cells to a greater extent than NCI-H146 cells (Fig. 5). Because each of these antibodies can inhibit aggregation of other cell types (Vestweber and Kemler, 1985; Patarroyo et al., 1987; Boyd et al., 1988; Shimoyama et al., 1989; Carter et al., 1990), we tested the effects of these antibodies on drug-induced aggregation of SCC-9 cells (Fig. 6). Aggregation of SCC-9 cells caused by carbachol or PMA is not altered by the control antibody nor by antibodies to ICAM-1 (Leu54) and α_3 -integrin (PIB5) (Fig. 6). However, antibodies to E-cadherin (DECMA and HECD) completely inhibit aggregation induced by either carbachol or PMA (Fig. 6). This strongly suggests that E-cadherin mediates drug-induced aggregation of SCC-9 cells. As a control experiment, we tested the ability of E-cadherin antibodies to inhibit PMA-induced aggregation of the human B cell line SA-4B, a process which is known to be mediated by ICAM-1 (Patarroyo et al., 1987). Antibodies to E-cadherin did not alter PMA-induced aggregation of SA-4B cells, whereas an antibody to ICAM-1 (Leu54) did inhibit aggregation (Fig. 7). This specificity of E-cadherin antibodies for inhibiting SCC-9 aggregation further supports the involvement of E-cadherin in drug-induced aggregation of SCC-9 cells. Additionally, we found that E-cadherin antibodies inhibit the compaction and morphological changes which SCC-9 cells undergo upon exposure to carbachol or PMA (Fig. 8).

Investigations of the Mechanism of E-cadherin-mediated Aggregation Induced by mAChR Activation or PMA Treatment

Having identified E-cadherin-mediated adhesion as the

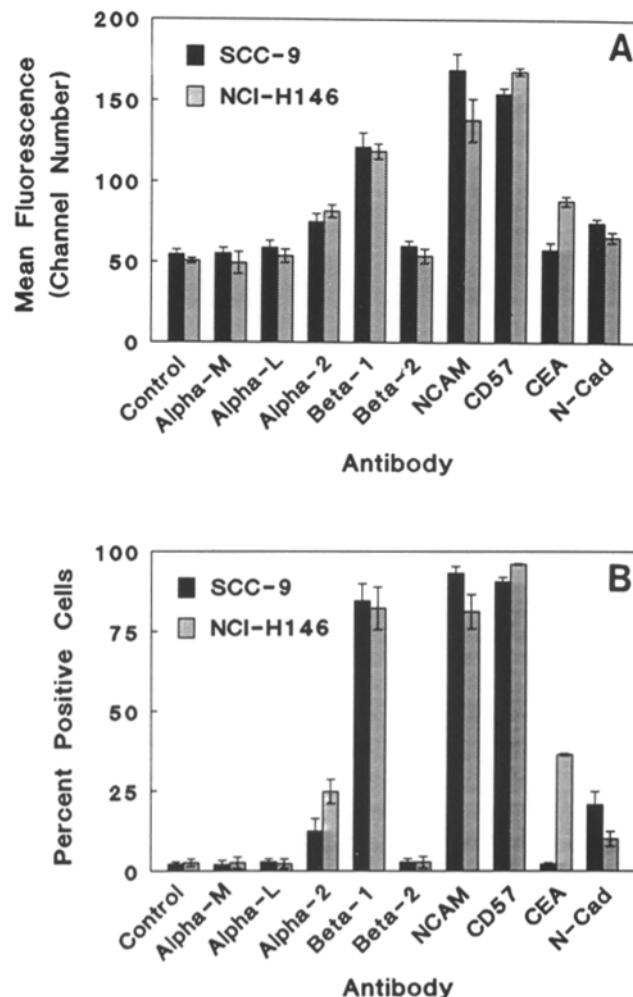


Figure 4. Identification of adhesion molecules which are expressed similarly by SCC-9 and NCI-H146 cells, or expressed to a lesser extent by SCC-9 cells. SCC-9 and NCI-H146 cells exhibit similar positive immunofluorescence when incubated with antibodies to α_2 integrin (*alpha-2*), β_1 integrin (*Beta-1*), NCAM (*NCAM*), the CD57 epitope (*CD57*), and N-cadherin (*N-cad*), indicating both cell lines express similar levels of these adhesion molecules. Antibody to CEA immunofluorescently stains NCI-H146 cells more than SCC-9 cells, indicating greater CEA expression by NCI-H146 cells. Immunofluorescent staining of SCC-9 and NCI-H146 cells by antibodies to α_M integrin (*alpha-M*), α_L integrin (*alpha-L*), and β_2 integrin (*Beta-2*) is equal to that produced by control antibody (*control*), indicating lack of expression of these adhesion molecules. Results shown are the mean (± 1 SEM) of three independent experiments.

event responsible for drug-induced aggregation, we next investigated possible mechanisms by which this process could occur. Carbachol or PMA may cause E-cadherin to localize at regions of cell-cell contact, resulting in aggregation and compaction. However, we observed that E-cadherin is concentrated at cell junctions in both drug-treated and non-treated cell populations, and there is no detectable gross change in localization due to drug treatment (Fig. 9).

Surface expression of E-cadherin may increase upon mAChR activation or PMA treatment. The aggregation and compaction of drug-treated cells precludes quantitative analyses of E-cadherin surface immunofluorescence using pho-

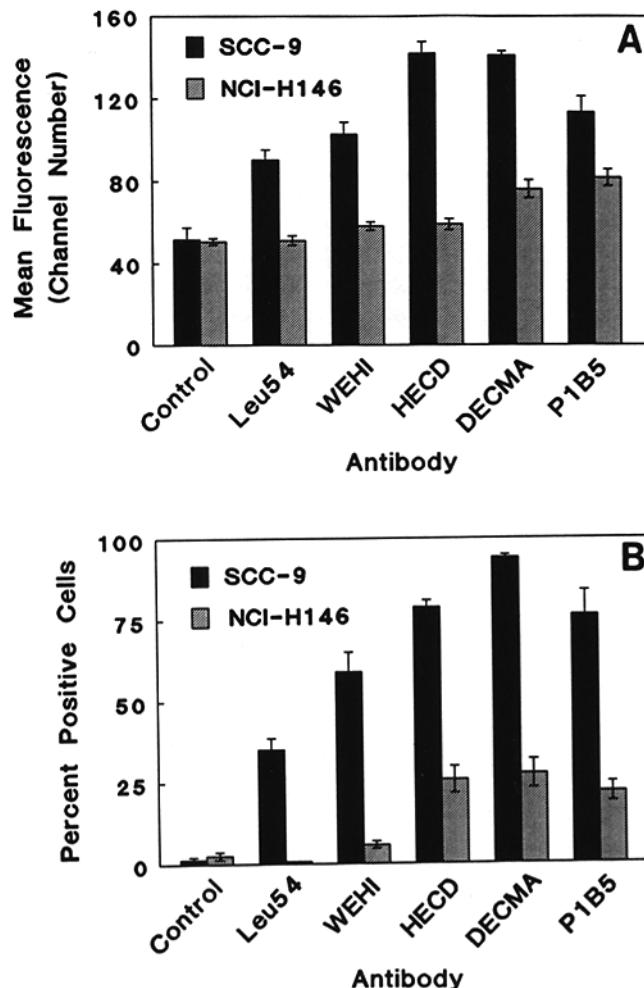


Figure 5. Identification of adhesion molecules which are expressed more by SCC-9 cells than NCI-H146 cells. SCC-9 cells are immunofluorescently stained more than NCI-H146 cells by antibodies to ICAM-1 (*Leu5⁴* and *WEHI*), E-cadherin (*HECD* and *DECMA*), and α_5 integrin (*PIB5*), indicating greater expression of these adhesion molecules by SCC-9 cells. Results shown are the mean (± 1 SEM) of three independent experiments.

tomicroscopy. Therefore, we used fluorescence-activated cell sorting to quantitate immunofluorescent surface staining by E-cadherin antibody. We found that immunofluorescent staining by E-cadherin antibody is unaltered or even slightly reduced in cells treated with carbachol or PMA (Fig. 10). This suggests that drug-induced aggregation is not due to increased surface expression of E-cadherin. Alternatively, some other alteration of E-cadherin, such as phosphorylation or association with cytoplasmic molecules may cause aggregation of drug-treated cells (see Discussion).

E-cadherin Expression in SCC-9 and Other SCLC Cell Lines

Although E-cadherin has previously been detected in biopsy specimens of SCLC using immunohistochemical techniques (Shimoyama et al., 1989), further characterization of E-cadherin in SCLC has not been reported. To characterize E-cadherin in SCC-9 and NCI-H146 cells, we immunoprecipitated E-cadherin from lysates of [³⁵S]methionine-labeled cells (Fig. 11). Negligible levels of E-cadherin were immunopre-

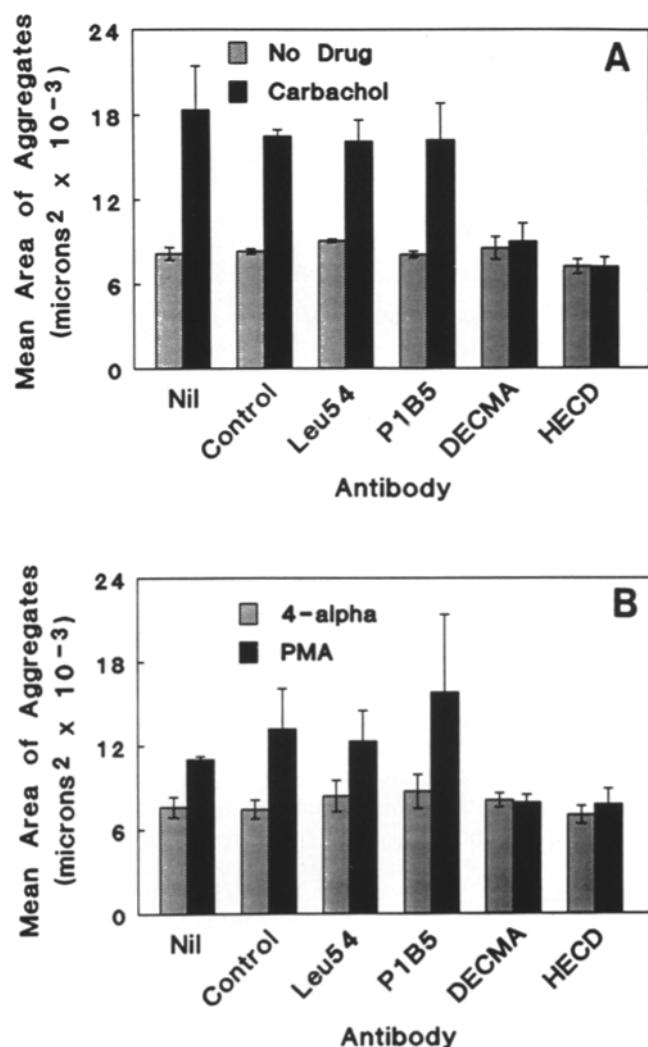


Figure 6. E-cadherin antibodies inhibit drug-induced aggregation of SCC-9 cells. The size of SCC-9 cell aggregates is increased by (A) carbachol (100 μ M, 1 h) or (B) PMA (60 nM, 1 h) in the absence of antibodies (*Nil*). This is not significantly altered by preincubating the cells with control antibody (*Control*) or antibodies to ICAM-1 (*Leu5⁴*) or α_5 integrin (*PIB5*). Drug-induced aggregation is completely inhibited by antibodies to E-cadherin (*DECMA* and *HECD*). Results shown are the mean (± 1 SEM) of three independent experiments. Matched pairs of *t* tests were used to determine significant differences. Other antibodies which were tested in this assay and found not to alter drug-induced aggregation include normal rat IgG (2 mg/ml) and NCAM antibody (10 μ g/ml).

cipitated from NCI-H146 cells, consistent with our immunofluorescence data. A protein with the molecular mass of E-cadherin (140 kD) as well as associated proteins with molecular masses of 109 and 97 kD were immunoprecipitated from SCC-9 cells lysed in buffer A (Fig. 11 A). Lysis of SCC-9 cells in buffer B yielded immunoprecipitates containing a 140-kD protein, as well as an associated protein with the molecular mass of 97 kD (Fig. 11 B). In immunoblots, E-cadherin antibody (HECD) recognized only the 140-kD protein and none of the other associated proteins (data not shown).

To investigate E-cadherin expression in other SCLC cell lines, we immunofluorescently stained several SCLC cell

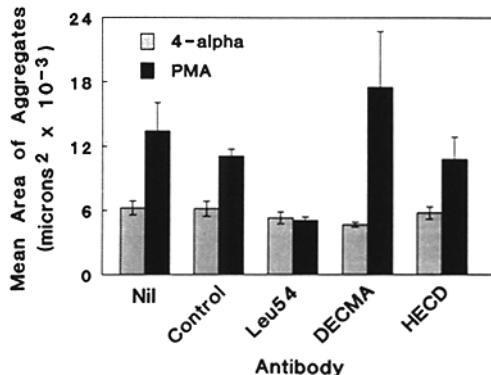


Figure 7. PMA-induced aggregation of SA-4B human B cells is inhibited by ICAM-1 antibodies, but not by E-cadherin antibodies. PMA (60 nM, 1 h) causes SA-4B cells to aggregate in the absence of antibodies (*Nil*). This is not significantly altered by control antibody (*Control*) or antibodies to E-cadherin (*DECMA* and *HECD*). PMA-induced aggregation is completely inhibited by ICAM-1 antibody (*Leu54*). Results shown are the mean (± 1 SEM) of three independent experiments. Matched pairs of *t* tests were used to determine significant differences.

lines with E-cadherin antibody. Although some of the SCLC cell lines we tested express significant levels of E-cadherin, none express levels as high as SCC-9 cells (Fig. 12). None of these other SCLC cell lines aggregate in response to PMA or carbachol.

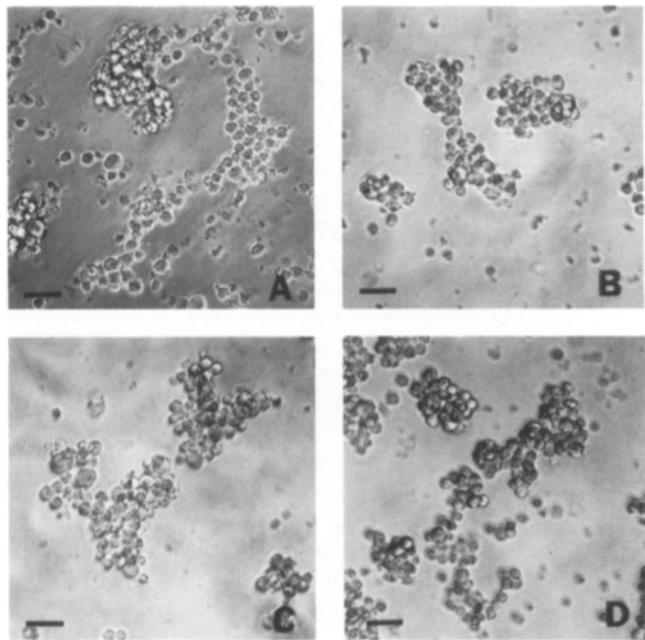


Figure 8. E-cadherin antibody inhibits drug-induced compaction of SCC-9 cells. SCC-9 cells were preincubated with E-cadherin antibody (DECMA, 1:5 dilution, 30 min) and exposed to (A) no drug, (B) carbachol (100 μ M, 1 h), (C) 4 α -phorbol (60 nM, 1 h), or (D) PMA (60 nM, 1 h). The cells do not compact and resemble the untreated cells shown in Fig. 2A. Similar results were obtained using the HECD antibody. Results shown are representative of three independent experiments. Bars, 50 μ m.

Discussion

Our results provide the first demonstration that activation of a heterologous, cell surface receptor can regulate binding activity of E-cadherin. Heterologous receptors regulate expression or activity of other adhesion molecules, such as β_2 -integrin (Dustin and Springer, 1989; Hibbs et al., 1991b), but no such receptor has previously been identified which regulates E-cadherin-mediated adhesion. Several lines of evidence indicate that mAChR activation increases cell-cell adhesion mediated by E-cadherin. First, carbachol-induced aggregation occurs only in SCC-9 cells, which have high E-cadherin expression. This does not occur in NCI-H146 cells, which have functional mAChR but express low levels of E-cadherin. Secondly, the compaction of SCC-9 cells induced by mAChR activation morphologically resembles compaction of mouse blastomeres, a process mediated by E-cadherin (compare Fig. 2C with Fig. 1 in Vestweber and Kemler, 1985, and Fig. 1 in Winkel et al., 1990). Finally, two different E-cadherin antibodies inhibit aggregation and compaction induced by mAChR activation in SCC-9 cells. The two antibodies which do this (DECMA, from rat, and HECD, from mouse) inhibit cell-cell adhesion mediated by E-cadherin in other systems (Vestweber and Kemler, 1985; Shimoyama et al., 1989). The specificity of these antibodies is indicated by their inability to alter ICAM-1-mediated adhesion of SA-4B human B cells.

We found that PMA mimics the effects of mAChR activation in inducing E-cadherin-mediated aggregation, suggesting that protein kinase C participates in this process. Activation of protein kinase C has previously been shown to induce E-cadherin-mediated compaction of mouse blastomeres (Winkel et al., 1990). Based on these results, Winkel et al. (1990) hypothesized that a cell surface receptor which is functionally coupled to protein kinase C may regulate E-cadherin-mediated compaction during embryonic development. Our demonstration that M_3 mAChR can regulate E-cadherin-mediated compaction of SCC-9 cells supports this hypothesis.

Recent studies indicate that pertussis toxin-sensitive G proteins may regulate cellular responses involving N-cadherin-mediated adhesion (Doherty et al., 1991). We found that pertussis toxin does not alter carbachol- or PMA-induced aggregation of SCC-9 cells, suggesting that pertussis toxin-sensitive G proteins are not involved in this response. This finding is consistent with the fact that M_3 mAChR-mediated responses are often regulated by G proteins which are insensitive to pertussis toxin (Hulme et al., 1990).

We found that E-cadherin expressed by SCC-9 cells has an apparent molecular mass of 140 kD, associates with accessory molecules, and is concentrated at cell junctions. These are similar to the characteristics of E-cadherin expressed by other cells. The molecular mass of E-cadherin has been reported to be 115–140 kD, depending on the species or cell type expressing the molecule (Vestweber and Kemler, 1985; Ozawa et al., 1989; Shimoyama et al., 1989; McCrea and Gumbiner, 1991; Nagafuchi et al., 1991). The proteins associated with E-cadherin in other cells include the α -catenins (α E-catenin and α N-catenin) at 102–103 kD (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989; McCrea and Gumbiner, 1991; Nagafuchi et al., 1991; Hirano et al., 1992), β catenin at 88–98 kD (Nagafuchi and Takeichi,

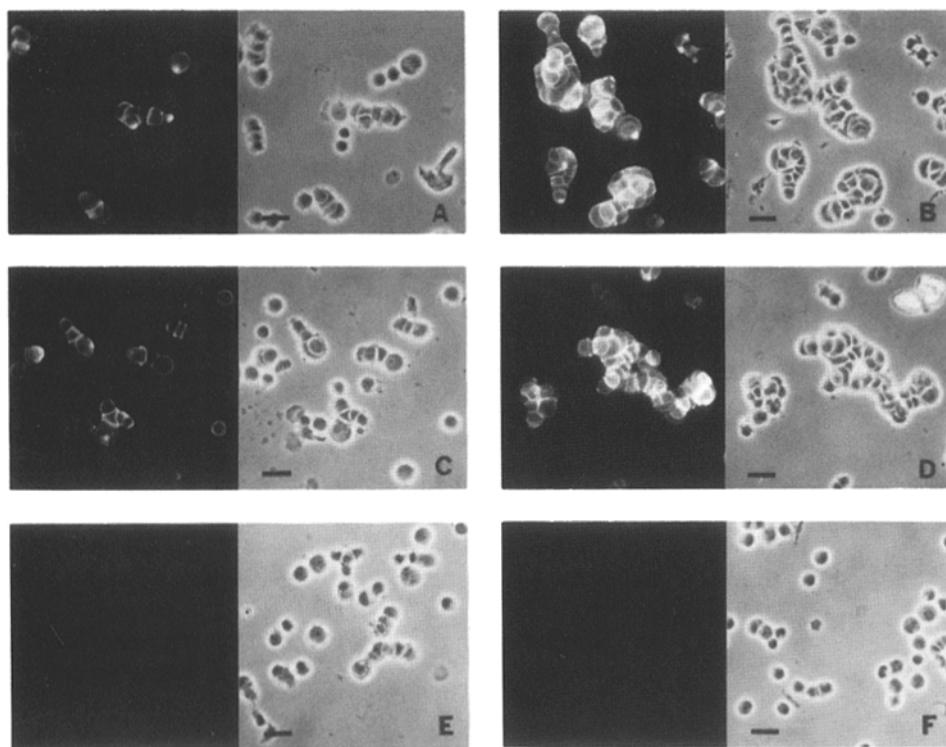


Figure 9. Localization of E-cadherin at SCC-9 cell junctions is not affected by mAChR activation or PMA treatment. The immunofluorescent localization of E-cadherin by HECD antibody at the junctions of SCC-9 cells (*A*) is not affected by (*B*) carbachol (100 μ M, 30 min), (*C*) 4 α -phorbol (60 nM, 30 min), or (*D*) PMA (60 nM, 30 min). Control antibody (*E*) does not immunofluorescently stain SCC-9 cells. NCI-H146 cells (*F*) are not immunofluorescently stained by HECD antibody. Results shown are representative of three independent experiments. Phase micrographs are shown on the right side of each figure. Bars, 30 μ m.

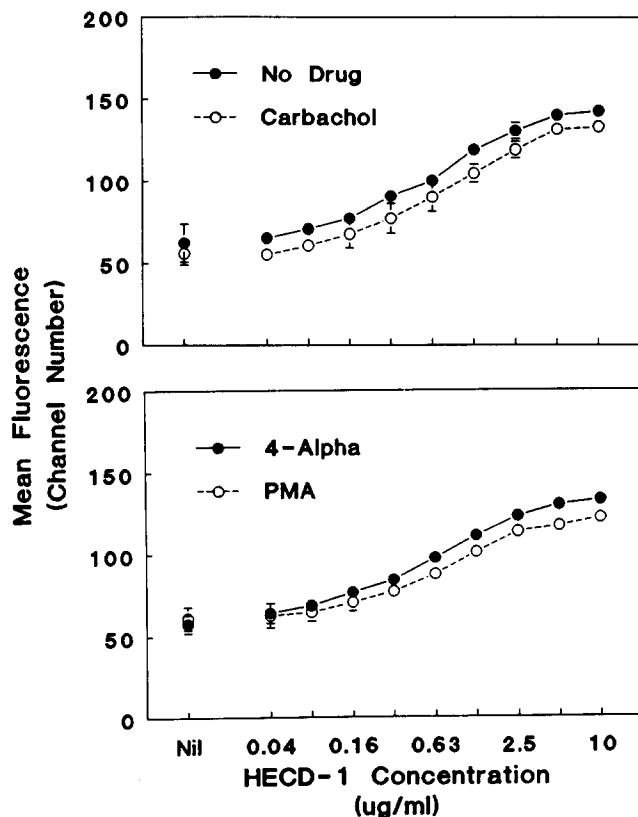


Figure 10. Surface expression of E-cadherin is not increased by mAChR activation or PMA treatment of SCC-9 cells. Immunofluorescent staining of SCC-9 cells by E-cadherin antibody (HECD) is not increased by carbachol (100 μ M, 40 min), 4 α -phorbol (4-alpha, 60 nM, 40 min), or PMA (60 nM, 40 min). Results shown are the mean (\pm SEM) of two independent experiments.

1989; Ozawa et al., 1989; McCrea and Gumbiner, 1991; Knudson and Wheelock, 1992; Peifer et al., 1992), and γ catenin and plakoglobin at 80–83 kD (Ozawa et al., 1989; Knudson and Wheelock, 1992; Peifer et al., 1992). Additionally, proteins of 85 and 78 kD associate with E-cadherin in other cell types (McCrea and Gumbiner, 1991). Similar proteins may associate with E-cadherin in SCC-9 cells, since proteins of 109 and 97 kD are immunoprecipitated from SCC-9 cells with E-cadherin antibodies. The types of proteins immunoprecipitated from SCC-9 cells depend on the lysis buffer used. This phenomenon has been observed in other systems (McCrea and Gumbiner, 1991) and may reflect the different abilities of the lysis buffers to disrupt the physical connection between E-cadherin and the associated proteins. The localization of E-cadherin at cell junctions in SCC-9 cells is similar to the localized expression of E-cadherin in other cells (Vestweber and Kemler, 1985; McNeill et al., 1990; Hirano et al., 1992; Knudson and Wheelock, 1992; Peifer et al., 1992).

We did not detect any gross changes in E-cadherin expression or surface localization in SCC-9 cells treated with carbachol or PMA. This suggests that some other modification of E-cadherin is responsible for drug-induced aggregation. Increased binding activity of E-cadherin, caused by changes in conformation or altered association with accessory molecules, may cause SCC-9 cells to aggregate upon mAChR activation or PMA exposure. This is consistent with the proposal by Winkel et al. (1990) that increased binding activity of E-cadherin may contribute to the compaction of mouse blastomeres exposed to PMA. In SCC-9 cells, E-cadherin binding activity may be increased by protein kinase C-mediated phosphorylation induced by mAChR activation or PMA treatment. Various protein kinases, including those activated by phorbol esters, phosphorylate other adhesion molecules,

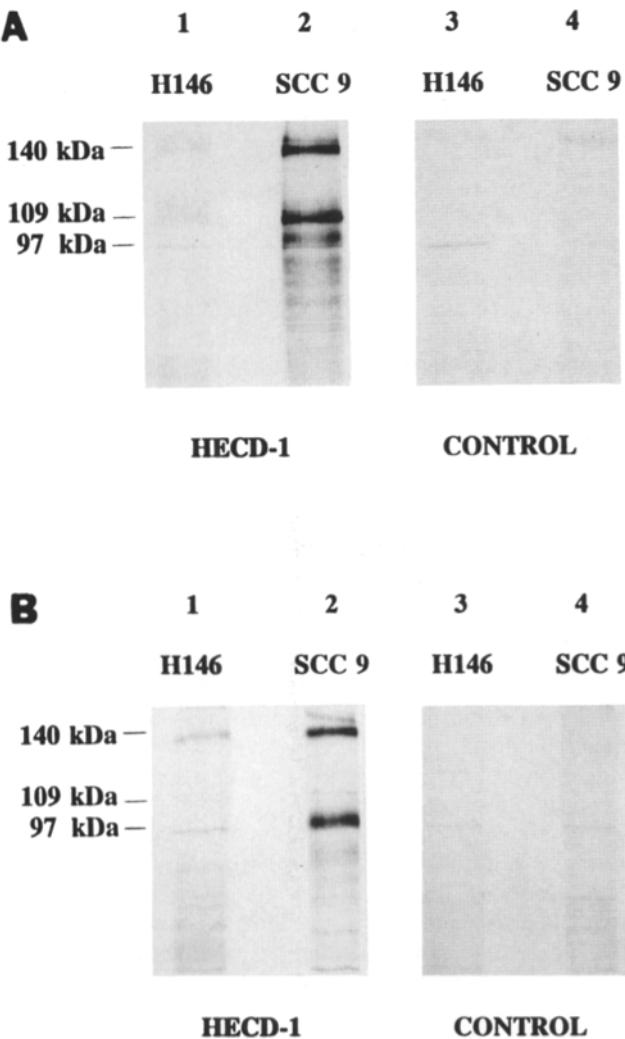


Figure 11. Immunoprecipitation of E-cadherin and associated proteins from small cell lung carcinoma cell lines. [³⁵S]methionine-labeled cells were lysed in buffer A (*A*) or buffer B (*B*) and immunoprecipitated with HECD antibody (lanes 1 and 2) or control antibody (lanes 3 and 4). NCI-H146 cells (lanes 1 and 3) express negligible levels of proteins specifically immunoprecipitated by HECD antibody. In SCC-9 cells (lanes 2 and 4), proteins specifically immunoprecipitated by HECD antibody include E-cadherin (140 kD) and associated proteins at 109 (*A*) and 97 kD (*A* and *B*). Results shown are representative of three independent experiments.

such as members of the integrin family (Hirst et al., 1986; Shaw et al., 1990; Haimovich et al., 1991; Hibbs et al., 1991a; Valmu et al., 1991). Both E- and N-cadherin are phosphoproteins (Cunningham et al., 1984; Lagunowich and Grunwald, 1991; Matsuyoshi et al., 1992; Sefton et al., 1992), and phosphorylation of E-cadherin and associated proteins correlates with altered cell adhesion (Matsuyoshi et al., 1992; Sefton et al., 1992). Because cytoskeletal associations may regulate the binding activity of E-cadherin (for review see Takeichi, 1991), phosphorylation of cytoskeletal proteins (Naka et al., 1983; Litchfield and Ball, 1986; Kawamoto et al., 1989; Leoncini et al., 1989; Beckerle, 1990) may also result in altered E-cadherin binding activity.

Drug-induced compaction of SCC-9 cells is transitory, declining after 4 h of continuous drug exposure. This is simi-

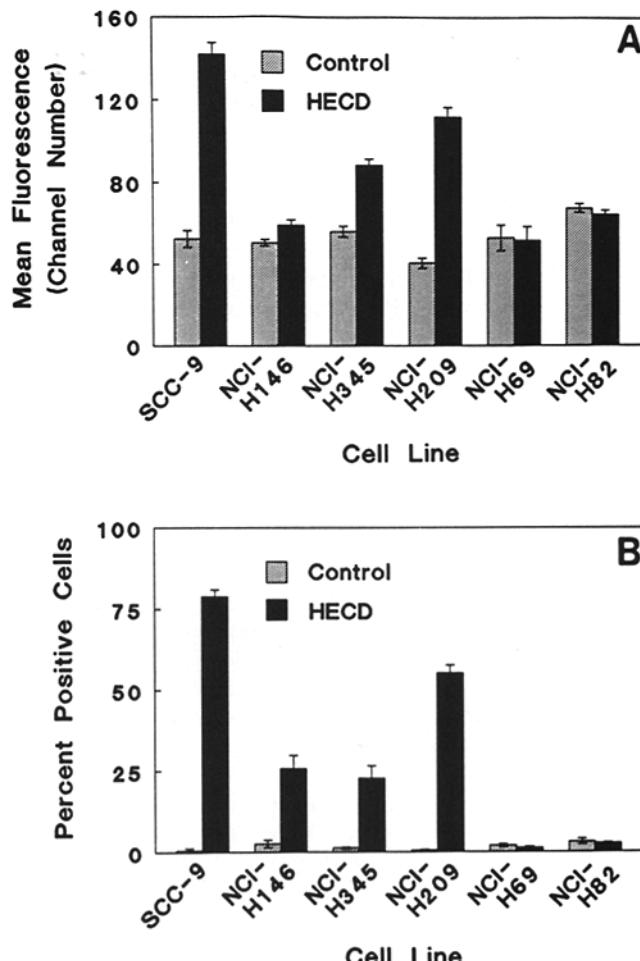


Figure 12. Surface expression of E-cadherin varies among different SCLC cell lines. E-cadherin antibody (HECD) immunofluorescently stains the SCC-9 cell line and, to a lesser extent, the NCI-H209 cell line. The NCI-H146 and NCI-H345 cell lines are less stained, whereas the NCI-H69 and NCI-H82 cell lines are not reactive with the HECD antibody. Results shown are the mean (\pm SEM) of three independent experiments.

lar to PMA-induced compaction of embryonic cells, which subsides after 1.5 h (Winkel et al., 1990). Downregulation of mAChR (Williams and Lennon, 1991) may account for decompaction of SCC-9 cells after prolonged mAChR activation. Similarly, downregulation of protein kinase C (Grove and Mastro, 1988) may cause decompaction of SCC-9 or embryonic cells incubated with PMA for extended periods.

Among the six SCLC cell lines we tested, only SCC-9 cells exhibit drug-induced, E-cadherin-mediated compaction. Low expression of E-cadherin probably accounts for the nonresponsiveness of most of these SCLC cell lines. However, NCI-H209 cells express fairly high levels of E-cadherin, but do not aggregate in response to carbachol or PMA. There may be several reasons for this. First, expression of E-cadherin by NCI-H209 cells may be below a threshold level needed for drug-induced compaction. It has previously been shown that cell-cell adhesion depends on the quantity of an adhesion molecule expressed at the cell surface (Friedlander et al., 1989). Secondly, an accessory molecule required for functional binding activity of E-cadherin may be absent in NCI-H209 cells. Hirano et al. (1992) re-

cently reported that E-cadherin-mediated adhesion is induced by transfecting α N-catenin in lung carcinoma PC9 cells, which express endogenous E-cadherin and β -catenin, but no α -catenin. Finally, the appropriate effector molecules needed for PMA-induced aggregation may not be expressed by NCI-H209 cells. For example, it was recently reported that SCLC cell lines express varying levels of protein kinase C isoforms, which have different substrate specificities (Baxter et al., 1992). If a particular isoform of protein kinase C participates in PMA-induced compaction of SCLC cells, those cell lines lacking this isoform would not respond.

Our finding that E-cadherin and other adhesion molecules are variably expressed among different SCLC cell lines is consistent with the results of other studies. Most SCLC cell lines express significant levels of $\alpha_3\beta_1$ integrin (Hemler et al., 1990; Feldman et al., 1991), NCAM (Kibbelaar et al., 1989; Patel et al., 1989; Aletsee-Utrecht et al., 1990; Rygaard et al., 1992), and the NCAM-associated epitope CD57 (Ball et al., 1986), similar to the cells lines we studied. More variable expression of ICAM-1 (Boyd et al., 1989), CEA (Goslin et al., 1983; Bepler et al., 1989), and N-cadherin (Rygaard et al., 1992) has been reported for different SCLC cell lines. A previous study demonstrated the expression of E-cadherin at cell junctions in SCLC biopsies taken from two independent cases (Shimoyama et al., 1989). The functions of these various adhesion molecules in SCLC cells are unclear. Expression of $\alpha_3\beta_1$ integrin may allow attachment of SCLC to the extracellular matrix (Hemler et al., 1990; Feldman et al., 1991). The roles of other adhesion molecules are less understood. For example, SCC-9 cells express ICAM-1 but do not express its ligands, $\alpha_4\beta_1$ integrin (Makgoba et al., 1988) or $\alpha_5\beta_1$ integrin (Diamond et al., 1990). These integrins are normally expressed by cells of the immune system (Makgoba et al., 1988). The reason why SCLC cells express a receptor for cells of the immune system is not readily apparent.

Modulation of adhesion molecule expression or activity may alter the metastatic and invasive capabilities of transformed cells. Several studies have demonstrated that adhesion mediated by E-cadherin restricts invasiveness of cells (Behrens et al., 1989; Chen and Obrink, 1991; Frixen et al., 1991; Vleminckx et al., 1991) and correlates with reduced metastatic potential (Hashimoto et al., 1989; Bussemakers et al., 1992; Ruggeri et al., 1992). Based on these findings, Vleminckx et al. (1991) suggested that drugs which increase cell adhesion by inducing E-cadherin expression may provide a new therapy for invasive and metastatic carcinomas. Our studies have identified another potentially therapeutic class of drugs which increase E-cadherin-mediated adhesion by activating cell surface receptors or second messenger pathways. These drugs could potentially affect metastasis in several ways. Increased cell adhesion may reduce metastasis by prohibiting the release of cells from a primary tumor mass. Modifying adhesion molecule activity may also change the location to which tumor cells metastasize. Tumor cells may preferentially metastasize to tissues which express adhesion molecules that bind the tumor cells (for review see Johnson, 1991; Zhu et al., 1991). Regarding this, it may be significant that the SCC-9 cell line was derived from a SCLC metastasis to the skin, which expresses E-cadherin (Wheellok and Jensen, 1992). If tumor cells have increased adherence for specific tissues, they may metastasize to these tis-

sues rather than those which are less adhesive for the tumor cells.

The roles of adhesion molecules in regulating neurite outgrowth (for review see Reichardt et al., 1989) and synapse formation (for review see Schubert, 1991) have recently been recognized. N-cadherin is widely expressed in the nervous system and may participate in neurogenesis (Matsunaga et al., 1988; Takeichi et al., 1990; Lagunowich et al., 1992). Other cadherins, including E-cadherin, are also present in the nervous system (Takeichi et al., 1990). Based on our finding that activation of a neurotransmitter receptor can increase cell adhesion, it is conceivable that adhesion of neuronal membranes may be stimulated by the binding of neurotransmitters. This hypothesis is attractive because it suggests a mechanism by which neurotransmitters may guide the migration of developing neurons, and promote the formation and maintenance of neuronal synapses. Several effects of activating mAChR in SCC-9 cells, such as decreased voltage-gated Ca^{2+} channel activity (Williams and Lennon, 1990) and altered proliferation (Williams and Lennon, 1991), also occur in neuronal cells upon mAChR activation (Wanke et al., 1987; Ashkenazi et al., 1989). Thus mAChR activation might stimulate adhesion of neuronal cells as it does SCC-9 cells. Evidence that synaptic adhesion may be regulated by neurotransmitters is provided by the recent demonstration that serotonin causes endocytosis of apCAM, an adhesion molecule related to NCAM, in sensory neurons of *Aplysia* (Bailey et al., 1992; Mayford et al., 1992).

Our finding that mAChR activation alters the binding activity of E-cadherin in SCC-9 cells has several broad-reaching implications. This system provides a novel model for investigating the regulation of cadherin-mediated adhesion. Unknown factors induce fluctuations in E-cadherin-mediated adhesion during embryonic development (Winkel et al., 1990; Sefton et al., 1992). Our results are consistent with the hypothesis that these fluctuations may arise due to signals transduced by cell surface receptors. Support for this is the remarkable similarity between embryonic blastomere compaction (Winkel et al., 1990) and mAChR-regulated compaction of SCC-9 cells. Both events are mediated by E-cadherin, probably involve protein kinase C, and temporally and morphologically resemble each other. It is possible that the phosphorylation of E-cadherin which occurs immediately before blastomere compaction (Sefton et al., 1992) may be due to activation of a cell surface receptor which functionally couples to protein kinase C. In analogy to SCLC cells which secrete autocrine growth factors (for review see Weynants et al., 1990), it is conceivable that embryonic cells may synthesize and secrete an autocrine factor which activates a cell surface receptor, inducing compaction. The regulation of cadherin-mediated adhesion by cell surface receptors may also play a role in other biological processes, such as morphogenesis and neurogenesis. Finally, because metastasis and invasion are intimately associated with cell adhesion, our studies suggest that modulating the activity of cell adhesion molecules by stimulating a cell surface receptor may be a viable approach for inhibiting metastatic disease.

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