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13. ABSTRACT ***(Maximum 200 Words)***

Tumor invasion is a major obstacle to effective clinical management of breast cancer. To identify new targets for anti-invasive therapies, we have focused on the mechanisms by which the cell adhesion molecule E-cadherin suppresses tumor invasion. A related cadherin, N-cadherin, does *not* suppress cell movement, even though it is as effective as E-cadherin at mediating adhesion. We have exploited this difference between E- and N-cadherin to define the region of E-cadherin required for suppression of movement. By constructing and analyzing a series of chimeric cadherins, consisting of parts of E- and N-cadherin, we localized the key region of E-cadherin to a region consisting of the transmembrane segment and a small portion of the cytoplasmic domain. We are analyzing components that bind specifically to this region. Further, we identified two components that are tyrosine phosphorylated after E-cadherin contact, but determined they play no role in suppression of motility.

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INTRODUCTION

Failures in treatment of breast tumors generally result from complications caused by tumor invasion and metastasis. This project aims to analyze mechanisms that regulate movement and invasion of mammary epithelial cells, with the ultimate goal of developing new anti-invasive therapies. One causal event in the acquisition of invasive capacity during breast tumor progression is loss of the cell-cell adhesion molecule, E-cadherin. We found previously that the ability of E-cadherin to suppress cell movement and invasion is not directly related to its adhesive activity. Instead, we hypothesize that cell-cell contact mediated by E-cadherin generates signals that suppress cell movement. We are testing this hypothesis and identifying components of the E-cadherin signaling system. We use molecular biological techniques to express different forms of E-cadherin in breast cancer cell lines, we analyze signaling events that are triggered by E-cadherin using biochemical approaches, and we test the effect of E-cadherin on cell behavior through time-lapse videorecording. In the past year, we have exploited the difference in ability to suppress motility between E- and N-cadherin to locate precisely the region of E-cadherin that is required. We have further shown that E-cadherin's effects on motility are not mediated by signaling involving either the EGF receptor or EphA2 receptor.

BODY

As described below, we have made substantial progress toward our overall goals. This year, however, some initial findings required us to make significant changes in our original plans. For this reason, we requested a no-cost extension to complete additional experiments that would fulfill the intent of the original objectives.

Our progress is concentrated in three major areas. First, we used the difference between E- and N-cadherin to define the region of E-cadherin involved in suppressing cell movement. Second, we decisively ruled out the possibility that E-cadherin affects motility solely in a passive way, by bringing together other receptors that do the actual signaling. Instead, we found that E-cadherin itself generates signals that affect motility. Finally, we identified two components that are tyrosine phosphorylated after E-cadherin contact and determined they play no role in suppression of motility. Unexpected results delayed our efforts to identify components that bind to the key region of E-cadherin.

SUMMARY OF WORK ACCOMPLISHED ON REVISED TECHNICAL OBJECTIVES

Technical Objective 1: To verify that E- and N-cadherin differ in their ability to suppress invasion of mammary carcinoma cells and to use this difference to define regions of E–cadherin that are essential for suppressing invasion.

Task 1. Months 1-3. We will assay the invasiveness of MDA-MB-435 cells and verify their N-cadherin expression.

> ***(Previously reported)*** *We verified the motility of our isolate of MDA-MB-435 cells and their expression of N-cadherin. For most of our experiments, however, we have used a different isolate of MDA-MB-435 that is motile and does not express N-cadherin, E-cadherin, or P-cadherin.*

Task 2. Months 3-9. MDA-MB-435 cells and MDA-MB-231 cells will be transfected with control, E-cadherin, N-cadherin (in the case of MDA-MB-231 cells), and chimeric E/N cadherin vectors and with E-cadherin mutant

vectors (for studies of Technical Objective 2). Permanent lines will be selected, re-cloned, and characterized for cadherin expression and adhesion.

*(**Previously reported**) We produced permanently transfected lines of both MDA-MB-231 and MDA-MB-435 that express full-length E- or N-cadherin and several cytoplasmic domain deletion forms of E-cadherin. Additionally, MDA-MB-435 cells were transfected with four E/N chimeric constructs and permanently transfected lines were obtained.*

*(**Year 3**) We completed the characterization of cadherin expression and adhesion of these lines. We also produced and characterized three new E/N chimeric cadherins, described below.*

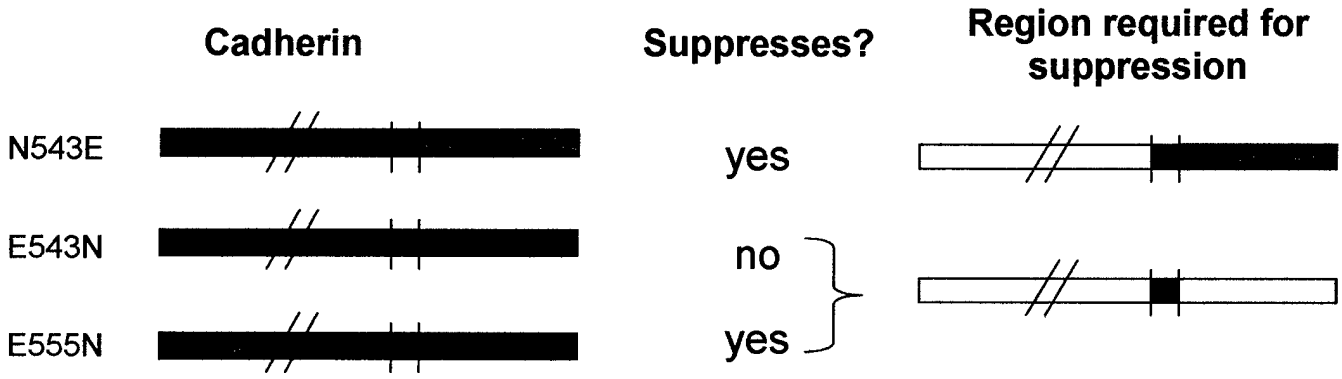
Task 3. Months 9-15. The invasion and motility of the transfected lines will be evaluated. Each assay will be performed 3-5 times.

*(**Year 3**) We completed assays of the motility of all the cell lines described under Task 2.*

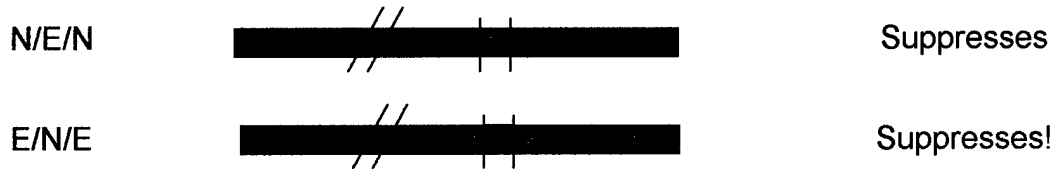
In in vitro assays, E- and N-cadherin are similar in adhesive activity, yet E-cadherin suppresses movement of MDA-MB-435 breast cancer cells, while N-cadherin does not. Thus, adhesion alone is not sufficient to suppress motility. Instead, E-cadherin must perform some additional function that N-cadherin does not. These findings ruled out the possibility that E-cadherin affects motility solely in a passive way, by bringing together other receptors that do the actual signaling. The results instead support the idea that E-cadherin itself generates one signal, which affects motility, and the adhesion mediated by E-cadherin enables interactions between other signaling molecules, which generate other signals that, however, have little effect on motility.

To define the region of E-cadherin required to suppress motility, we began constructing a series of E/N chimeric cadherins (Objective 1, tasks 2 and 3). For each of the MDA-MB-435 cells transfected with expression vectors for the chimeric cadherins, multiple, independent clones with similar levels of cadherin expression were examined in the wound-filling assay with consistent results. Results reported last year suggested that the transmembrane domain is the key region of E-cadherin.

In Year 3, analysis of an additional construct (E555N), shown below with some key comparisons, confirmed this conclusion:



We tested this conclusion further by constructing and expressing two more chimeric cadherins (see below), one in which the transmembrane domain of N-cadherin was replaced with an E-cadherin segment and vice-versa. As expected, the N/E/N chimera did suppress motility. We were very surprised, however, to find that the E/N/E chimera also suppressed motility!



The unexpected nature of these findings is underscored by the following summary comparison:

Cadherins	Transmembrane segment	Cytoplasmic segment	Suppression?
E-cadherin, N543E	E	E	Yes
E555N, N/E/N	E	N	Yes
N555E, E/N/E	N	E	Yes
N-cadherin, E543N	N	N	No

The transmembrane and cytoplasmic segments of N-cadherin each permit suppression when combined with E-cadherin, but not when combined with each other! While these findings seem almost self-contradictory, it should be emphasized that these results were consistently obtained with several independently derived cell lines.

These newest results required significant alterations in our experimental plans. When we first identified the transmembrane domain as important, we initiated a collaboration with Dr. Dieter Langosch of the University of Heidelberg, an internationally recognized investigator in the field of transmembrane domain interactions. Dr. Langosch's lab had shown previously that the E-cadherin transmembrane segment contributes to E-cadherin dimerization and that the E- and N-cadherin transmembrane segments differ significantly in dimerization capacity. Thus, we began a collaboration to investigate whether differences in dimerization of E- and N-cadherin could be responsible for the differences in effect on motility. The finding that her transmembrane domain of E-cadherin could be replaced with the transmembrane segment of N-cadherin without affecting suppression (compare E-cad vs. E/N/E) indicated that differences in dimerization capabilities of the transmembrane segment could not be the basis for the difference between E- and N-cadherin. The collaboration was regretfully discontinued, therefore.

One interpretation of these unusual findings is that some unidentified component binds to the N/N region and prevents N-cadherin from suppressing motility. This component would not bind to E/E or to the combination of sequences found in E/N or N/E and thus would permit these molecules to suppress. One candidate protein was the src-family tyrosine kinase fer, recently shown by Jack Lilien's group to bind to N-cadherin, employing sequences adjacent to the plasma membrane. We have collaborated with Dr. Lilien to

test this hypothesis, but found that fer binds equally to E-cadherin and non-suppressing E/N chimeric cadherins.

Technical Objective 2 To determine whether an intact juxtamembrane domain is required for E-cadherin-induced tyrosine phosphorylation.

(Year 3) In light of earlier findings, this objective was modified to test whether the critical region of E-cadherin (the transmembrane/juxtamembrane region defined as required for suppression) is necessary for initiating a tyrosine phosphorylation cascade

Task 1. Months 4-10. Work out assays and then complete final analyses of tyrosine phosphorylation in untransfected MDA-MB-435 cells and MDA-MB-231 cells.

Task 2. Months 12-24. Assay tyrosine phosphorylation in MDA-MB-435S cells *and MDA-MB-231 cells* transfected with full-length or mutant E-cadherins.

(Year 3) We found evidence that E-cadherin-mediated contact initiates several parallel cascades of tyrosine phosphorylation. One pathway involves activation of the EphA2 receptor tyrosine kinase and another pathway involves transient activation of the EGF Receptor. By comparing receptor activation with suppression of motility in the panel of cell lines expressing different chimeric cadherins, we were able to prove that neither of these pathways is required for suppression of motility.

Technical Objective 3 To identify factors that interact with the juxtamembrane domain of E–cadherin.

This objective was modified as the critical region of E-cadherin was defined.

(Task 1 and Task 2. *These experiments were duplicative of the NIH award and were deleted).*

Task 3. Months 4-10. We will analyze, by co-immunoprecipitation studies, components that may be associated with E-cadherin, but not the JM-deleted form, in MDA-MB-435 cells and MDA-MB-231 cells.

(Year 3) We have previously reported that preliminary experiments using this approach were unsuccessful and alternative approaches were initiated. However, these alternative approaches were rendered useless by the unexpected discovery that the transmembrane segment is crucial to the suppressive activity of E-cadherin. We have, therefore, focused more effort on this original approach and have obtained encouraging preliminary results.

*As shown in Figure 1 on the following page, we are using immunoprecipitation with specific antibodies, followed by 2D gel analysis, to compare the pattern of proteins associated with E-cadherin or an E/N chimeric cadherin that does not suppress motility. Many of the spots seen were common to both cadherins or were found in control, preimmune serum precipitates. These 2D gels reveal one low molecular weight component (marked by +) found in both immunoprecipitates that has not previously been reported to be associated with cadherins. More important, two components (marked by *) appear to be associated with E-*

but not E/N-cadherin. We are currently repeating these analyses and attempting to identify these components by preparing amounts adequate for mass spectrometry analysis.

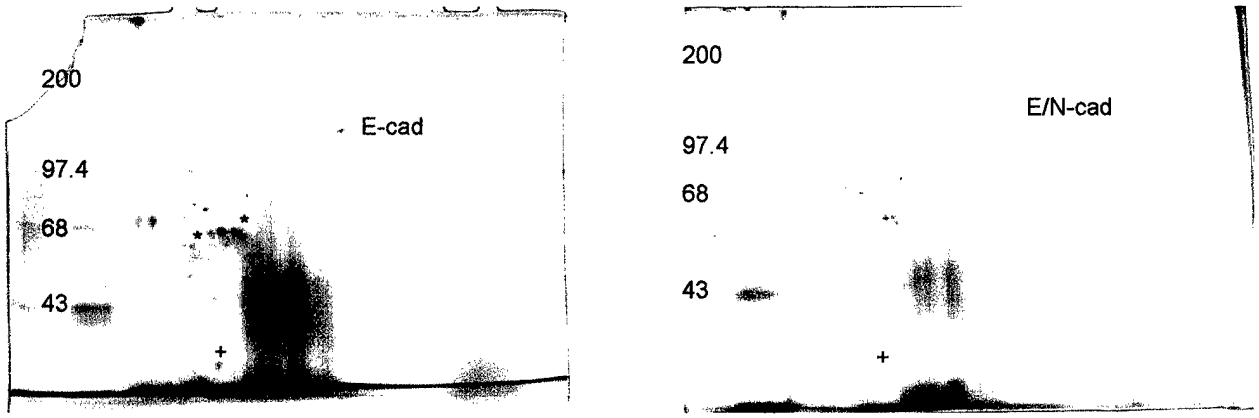


Figure 1. Proteins associated with E-cadherin and an E/N chimeric cadherin. Extracts of MDA-MB-435 cells transfected with expression vectors for E-cadherin (left panel) or an E/N chimeric cadherin that does not suppress motility (right panel) were extracted and specifically immunoprecipitated with polyclonal antibodies to E-cadherin. The immunoprecipitates were solubilized and analyzed by two-dimensional gel electrophoresis. The first dimension (left to right) was isoelectric focusing (ampholine range pH 3-10) and the second (vertical) dimension was SDS-PAGE. Molecular weight markers (shown in kDa) were added for the second dimension. Proteins were visualized by silver staining. The E-cadherin and E/N cadherin spots are labeled. The large smear in the lower center of the gel is the antibody heavy chain. Several other spots were also seen in control, pre-immune precipitates (not shown). One low molecular weight spot (marked by +) was seen in both panels. Two other spots (marked by *) were present in the E-cadherin immunoprecipitates, but largely absent from the E/N cadherin immunoprecipitates.

Tasks 4 (Months 10-24)**, 5** (Months 6-15)**, and 6** (Months 16-36)**.**

No work yet initiated.

Task 7. Months 9-18. We will use GST-fusion proteins for affinity-purification of components associated with the JM domain.

This experimental approach is not applicable for the region we defined by analyzing the chimeric cadherins. Therefore early attempts that we had initiated were later abandoned.

Task 10. Months 18-24. We will produce antibodies against the affinity-purified components and begin testing for physiological interactions.

No work yet initiated.

Task 11. Months 18-36. We will attempt to identify proteins that are tyrosine phosphorylated in response to E-cadherin binding and will begin to test whether they interact with E-cadherin.

(Year 3) We have identified two such proteins, the receptor tyrosine kinases EphA2 and EGF Receptor. We confirmed the observations of others that EGFR interacts with E-cadherin, and showed for the first time that the interaction is via their extracellular domains. EphA2 does not interact with E-cadherin. Analysis

of the panel of cell lines expressing chimeric cadherins indicated, however, that there was no correlation between suppression of motility and activation of either EphA2 or EGFR, so neither of these proteins is required for suppression of motility by E-cadherin.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that E- and N-cadherin differ in ability to suppress movement of mammary epithelial cells
- Localized the region of E-cadherin required for suppression of movement to a region including the transmembrane domain and a small segment of the cytoplasmic domain.
- Showed that cadherin-mediated cell-cell adhesion is required for activation of EphA2 by its ephrin ligands and that this activation reduces focal adhesions, but is not necessary for suppression of cell movement.
- Showed that E-cadherin regulates activation of EGFR by ligand and does so by interactions via the extracellular domains of the two molecules. This activation reduces focal adhesions but, again, is not required for suppression of cell motility.

REPORTABLE OUTCOMES (Year 3)

Publications:

Hein, P., Chaiken, M., Stewart, J.C., Brackenbury, R., and M. S. Kinch (2001). E-cadherin Binding Regulates EGF Receptor Activation. *J. Cell Science*, currently in revision.

New Cell Lines

In addition to lines previously reported, we have produced additional variants of MDA-MB-435 transfected with expression vectors encoding additional chimeric E- and N-cadherins (as described in Technical Objective 1, task 3).

CONCLUSIONS

Cell-cell contact mediated by E-cadherin suppresses movement of mammary epithelial cells. We have demonstrated that it is not the adhesive activity, but more likely a signaling activity of E-cadherin that regulates cell movement. We showed that N-cadherin does not suppress motility and used the difference between these two molecules to define the region of E-cadherin that is required. The results suggest that a region encompassing the transmembrane segment and a small portion of the cytoplasmic domain is crucial. We are currently working to identify proteins that interact with this region, which may be useful diagnostic or therapeutic targets for invasive tumors.