Haik Mkhikian believes his undergraduate research experience is the primary reason for his award of a National Institutes of Health (NIH) scholarship. Haik will graduate this spring with a double major in Biochemistry and Molecular Biology, and Philosophy. After graduation, he will research in a laboratory at NIH for a year as he submits applications for medical school. Haik credits his best learning experiences to his failed experiments, which forced him to think of new approaches to his research. He counsels students to seek challenges and not to settle for easy classes. In his free time, Haik reads Indian philosophy and plays basketball, ping pong, and hacky sack.

There is increasing understanding that tumor cells are not always aggressive solely because of their own characteristics. In many instances the normal tissues that surround tumor cells contribute to their behavior. Haik Mkhikian’s work is significant because it shows that normal breast cells can lead to one of the earliest steps of cancer spread—the separation of tumor cells from their surroundings. In this case, the separation is mediated by decreased expression of E-cadherin. In normal cells, E-cadherin acts as a “molecular glue” that keeps cells together. This work illustrates how the efforts of a dedicated student can lead to novel scientific findings.
Introduction

Cadherins are glycoprotein cell-cell adhesion molecules found in the membranes of vertebrate cells. Cadherins mediate binding between cells through calcium-dependent homotypic interactions (Pignatelli and Vessey, 1994). The most studied and best characterized cadherin is endothelial cadherin (E-cadherin), which is found on the cell membranes of epithelial cells.

A key characteristic of aggressive malignant cancer is its ability to metastasize and invade distant tissues and organs. Metastasis is the separation of individual cancer cells from the main tumor and their subsequent migration through tissue to a blood vessel that will carry them to distant sites (Liotta et al., 1991; Cotran et al., 1994; Fidler and Ellis, 1994). Such motility in cancer cells has been associated with decreased levels of E-cadherin expression (Shiozaki et al., 1991; Moll et al., 1993; Maguire, et al., 1997). Shiozaki and his colleagues found that intercellular adhesion is destabilized in a significant number of cancer cells.

Cancer cell motility can be instigated by a number of proteins referred to as motility factors (Stoker and Gherardi, 1991). Such motility factors have been shown to promote the movement of cancer cells away from the main tumor and toward blood vessels. When cancer cells are exposed to proteins secreted by normal breast cells, they often dissociate and exhibit the type of motility seen in cancer (Carpenter and Nguyen, 1998).

Though it is now established that motility factors promote the movement of cancer cells in tumors and, furthermore, that some motile cancer cells exhibit decreased levels of E-cadherin expression, the direct link between motility and cell separation has not yet been demonstrated. In an effort to better understand the mechanisms by which motility factors (specifically, those found in the secretions of normal breast cells) are able to promote cancer cell separation, we conducted a set of experiments aimed at elucidating the connection between motility factor function and its effects on E-cadherin levels. We hypothesized that the separation observed in tumor cells as a result of their contact with normal breast cell secretions is due to a loss of E-cadherin function in the cells and, therefore, that normal breast cell secretions act to downregulate E-cadherin in breast cancer.

To test our hypothesis, we conducted three types of experiments. First, we examined/explored/analyzed the effect of conditioned medium treatment (treatment with medium containing secretions from normal breast cells) on E-cadherin staining of MCF-7 human breast cancer cells. This experiment allowed us to also compare the results and morphology of the cells with the staining and further correlate E-cadherin staining with motility. Second, we quantified the effects of conditioned medium (CM) treatment on E-cadherin levels with flow cytometry. Cells were treated with a primary antibody specific for E-cadherin and then with a secondary antibody coupled with a fluorescent dye. The cells were then passed through a flow cytometer to measure their degree of brightness (signal intensity). If E-cadherin is downregulated by treatment with CM, then we would expect the flow cytometer to detect a lower degree of fluorescence for the CM-treated sample than for a sample in which E-cadherin expression is normal. Finally, we conducted a cell-cell binding assay wherein we measured the ability of the treated cells to bind to a monolayer of MCF-7 cells. We expected lower levels of E-cadherin to translate to a decrease in intercellular binding.

Materials and Methods

Tissue Culture

Tissue culture medium components and other chemicals were obtained from Sigma Chemicals (St. Louis, MO) unless indicated otherwise. MCF-7 cells were a gift from Dr. Dan Mercola (Sidney Kimmel Cancer Center, San Diego, CA). 184A1 cells have been well characterized and share many qualities with parental normal cells, including anchorage dependence, non-tumorigenicity, and epidermal growth factor (EGF)-dependent growth (Stampfer and Yaswen, 1993).

MCF-7 cell medium consisted of RPMI 1640 medium supplemented with 4 mM glutamine, 0.2 U/mL bovine insulin, 10 U/mL penicillin, 10 µg/mL streptomycin, 5% heat-inactivated newborn calf serum (Irvine Scientific, Santa Ana, CA), and 10 nM estradiol. MCF-7 GFP(++) cell medium contained 250 µg/mL geneticin in addition to regular MCF-7 medium. 184A1 cells were routinely grown in Mammary Epithelial Growth Medium (MEGM) (Clonetics, San Diego, CA). MEGM consists of modified MCDB 170 medium supplemented with approximately 52 µg/mL bovine pituitary extract, 10 ng/mL EGF, 0.5 µg/mL hydrocortisone, 5 µg/mL insulin, 50 µg/mL gentamicin sulfate, and 50 ng/mL amphotericin B.

Production of Conditioned Medium

CM was collected from 70-100%-confluent plates of 184A1 cells. To minimize the amount of exogenous protein in the CM, the MEGM used to establish the plate was discarded. The monolayer was rinsed with phosphate buffered saline (PBS) and the medium was replaced with RPMI without
serum. Under these conditions, the cells remained viable and secreted proteins into the medium. The CM was passed through a filter with 2 µm pores and was concentrated to 6x by ultrafiltration through a YM30 filter in a Centricon plus bioconcentrator apparatus (Millipore, Bedford, MA), which retained proteins with molecular weights of 30 kD or more.

**E-cadherin Staining**

2500 MCF-7 cells in 1 mL of medium were added to each well of two four-well chamber slides. To each well of the chamber slides, varying doses of 6x concentrated 184A1 CM were added to final concentrations of 0%, 1%, 5%, and 20% in MCF-7 medium. The cells were incubated in these solutions at 37 °C for 48 hr. The medium and the walls of the wells were removed, and the slides were rinsed with PBS and fixed in 95% ethanol. Each well of one slide was incubated with 100 µL of 20% anti-E-cadherin antibody (commercially available, pre-diluted HECD-1; Zymed Laboratories Inc., South San Francisco, CA) in antibody buffer (0.15 M NaCl, 10 mM Tris, pH 7.4) for 1 hr. As a negative control, each well of the other slide was incubated with 10 µg/mL mouse IgG (BioGenex, San Ramon, CA) in antibody buffer for 1 hr. Each well was then incubated with two drops of LINK (biotinylated anti-mouse immunoglobulins, BioGenex) for 20 min. Wells were rinsed again with antibody buffer and incubated with two drops of LABEL (peroxidase-conjugated streptavidin, BioGenex) for another 20 min. After rinsing with antibody buffer, wells were incubated in the dark with 3-4 drops of diaminobenzidine (DAB) solution (0.33 mg/mL DAB in 0.05 M Tris, pH 6.3; 0.05% hydrogen peroxide) for 10 min. Slides were rinsed with DI water, counterstained, and examined under a light microscope.

To quantitate our findings, we counted the number of stained cells from three random clusters per well. Each well was marked randomly three times with a pen. The cluster of cells between 10 and 80 cells closest to each pen mark was counted. Cells were considered motile if they had separated from the main cluster on at least three out of four sides. Cells within the cluster were said to have continuous staining if they had DAB reaction product on all sides of the cell. Additionally, cells on the periphery were said to have continuous staining if DAB staining appeared on all sides except those facing the outside of the cluster. Cells were deemed partially stained if they had E-cadherin staining on at least one side or section of their membranes (including continuously stained cells).

**Flow Cytometry**

3 x 10^6 MCF-7 cells in 5 mL of medium were added to two 100 mm plates. One plate was treated with 1 mL of 6x concentrated 184A1 CM and the other with 1 mL of RPMI 1640. The plates were then incubated for 48 hr at 37 °C after which the cells were removed from the plates with 2 mL of 2 mM EDTA in PBS. Cells that were dislodged from the plate were pipetted to separate the clusters and then diluted with 8 mL of PBS. The solutions were centrifuged at 1000 rpm for 10 min and the top 8 mL of each was discarded. The cells in each tube were suspended again in the remaining 2 mL of solution and separated into two fractions (experimental and control). Each experimental fraction was incubated for 20 min at 4 °C with 2% HECD-1. Control fractions were incubated in 1 µg/mL mouse IgG. After the incubation period, the solution from each fraction was removed and rinsed with PBS. All four fractions were then incubated in 1% labeled anti-mouse Ig (PE conjugated 2° antibody, Chemicon, Temecula, CA) at 4 °C for 20 min. The solutions were again removed and the fractions were rinsed with PBS. Each fraction was then immediately analyzed by the flow cytometer.

**Cell Binding Assay**

Fluorescent MCF-7 GFP(++) cells (MCF-7 cells transfected with green fluorescent protein (GFP)) were placed into a single cell suspension at 100,000 cells/mL and separated into two fractions. The fractions were incubated in either 16.7% 6x concentrated CM solution or an equal concentration of RPMI 1640 at 37 °C for 48 hr. Additionally, a monolayer of regular MCF-7 cells was set up in 12 wells of a 24-well plate. Six of the 12 wells were treated with 16.7% 6x concentrated CM, whereas the other six were treated with an equal amount of RPMI 1640. The plate was incubated at 37 °C for one day. The single cell suspension and monolayer were set up so that their respective incubation periods ended at the same time. At this point, the GFP(++) cells were removed from the plates with 2 mM EDTA and brought to 100,000 cells/mL with medium. To each fraction, 2 µL of CaCl_2 was added to counteract the effects of the EDTA on E-cadherin. Then, 10,000 of the CM-treated GFP(++) cells were added to each of the six CM-treated wells of the monolayer plate, and 10,000 of the untreated cells were added to the six untreated wells of the plate. The plates were then incubated for 30 min and three plates from each of the treated and untreated wells were rinsed with PBS and replaced with medium. The plates were incubated overnight, rinsed with PBS, and examined under a fluorescent microscope. The fluorescent cells in 10 random 10x fields were counted for each well and recorded. Percent binding was calculated as the ratio of bound cells in the rinsed wells over bound cells in the unrisened wells. P values were calculated using the unpaired t-test.
**Results**

_E-cadherin Staining Showed a Dose-Dependent Decrease in _E-cadherin_ Levels_

The staining procedure gave a visual account of the CM’s effect on E-cadherin. With no CM added, the cells showed distinct staining at the interface between two cell membranes (Figure 1A). The cytoplasmic staining of the cells also appeared relatively dark. However, as the dose of CM added increased to 20%, the staining decreased both at the membrane interfaces and throughout the rest of the cell (Figure 1B). As is apparent in Figure 1, increasing the dosage of CM resulted in a lower number of stained cells as well as an overall paler appearance of the cluster as a whole. We quantified both the number of continuously-stained cells and partially-stained cells and noted a marked decrease in both as CM was added. Additionally, we observed a corresponding increase in cell motility from less than 5% of the cells in the cluster in the absence of CM to nearly 80% motility at 20% CM. As shown in Figure 2, a dose-dependent decrease in staining corresponded with a dose-dependent increase in motility.

**CM Treatment Resulted in a Quantitative Decrease in _E-cadherin_ Levels by Flow Cytometry**

We had four fractions for this experiment: two negative controls, a positive control, and an experimental fraction. The CM-treated and untreated fractions that were incubated with mouse IgG instead of HECD-1 served as the negative control and showed minimal fluorescence, as expected. The CM-untreated fraction that was incubated with HECD-1 as the primary antibody served as the positive control and exhibited about 90% fluorescence. In comparison, the experimental fraction, which was treated with 6x 184A1 CM and incubated with HECD-1, showed only 68.2% fluorescence in the same region. This reduced fluorescence of the CM-treated cells indicates that less _E-cadherin_ was present on their cell membranes compared to the cell membranes of untreated cells (Figure 3).

**CM Treatment Resulted in Decreased Intercellular Binding**

Since MCF-7 GFP(++) cells were fluorescent whereas normal MCF-7 cells were not, the cells added to the monolayer were distinguishable with the use of a fluorescent microscope. In this manner, we were able to quantify the number of cells bound to the monolayer and determine the effect of CM on cell-cell binding. Our data showed 46.1% binding between CM-treated GFP(++) cells and the CM-treated monolayer versus 72.7% binding between the untreated GFP(++) cells and untreated monolayer (Figure 4). The decrease in binding was determined to be statistically significant (p < 0.005).
Data from E-cadherin staining, flow cytometry, and cell binding assays provided three avenues of insight into the relationship between motility factors secreted by normal breast tissue and the expression of E-cadherin in MCF-7 cancer cells. Overall, our data provide evidence that certain factors secreted by 184A1 cells act to downregulate E-cadherin in cancer cells. The dose-dependent response in the staining experiment showed a strong correlation between increased motility and decreased E-cadherin staining, suggesting that motility factors decrease cell-cell adhesion molecules present on cell membranes of cancer cells. Quantitative data from flow cytometry provided further support for our hypothesis. Cell binding assays also confirmed previous data by showing that treatment of the cells with CM results in a direct decrease in intercellular binding. Hence, we found that normal breast tissue secretions act to downregulate E-cadherin in cancer cells.

Despite the fact that our data supported our hypothesis, they do not suggest a complete or even a drastic decrease in E-cadherin as compared to the marked increase in motility that is seen in correspondence. Only a 20-25% decrease in binding and fluorescence was found as compared to an 80% increase in motility. The large decrease in E-cadherin staining is also questionable because E-cadherin tends to be concentrated at points where the cell membranes of two cells are in contact and, in general, cadherin-cadherin interactions cause a rise in E-cadherin levels (Conacci-Sorrell et al., 2003). Thus, if motility were caused by some other mechanism, we might still expect to see less staining in detached cells. However, the dramatically paler appearances of the CM-treated cells in addition to our other data suggest that there is an authentic decrease in E-cadherin in these cells. Furthermore, a comparatively small decrease in E-cadherin may be enough to allow many cells to dissociate and thus lead to a high increase in percent motility. An interesting result is the presence of staining near the nucleus of CM-treated cells, which complements prior work from our lab and suggests redistribution of E-cadherin from the cell membrane to the cytoplasm (Carpenter et al., 2002).

Downregulation of E-cadherin is most likely not the only factor resulting in cell separation. Nonetheless, the cell-cell adhesions must be deactivated or at least destabilized to allow for motility. Previous research has shown that decreases in catenin levels also act to weaken the E-cadherin-mediated intercellular binding (Hirohashi, 1998; Ino et al., 2002). Since catenins help to anchor E-cadherin to the actin cytoskeleton of the cell, their downregulation would also contribute to decreased intercellular adhesion (Mareel et al., 1997). Thus, motility factors in CM may also act to promote motility by downregulating cellular catenin levels, or by causing a dissociation of either the E-cadherin-catenin or catenin-cytoskeleton complexes. Another possibility is that the motility factors deactivate the E-cadherin in addition to downregulating it. Future studies could test for the effect of CM on the functionality of E-cadherin as the only adhesive molecule that can result in cell binding.

Discussion

Data from E-cadherin staining, flow cytometry, and cell binding assays provided three avenues of insight into the relationship between motility factors secreted by normal breast tissue and the expression of E-cadherin in MCF-7 cancer cells. Overall, our data provide evidence that certain factors secreted by 184A1 cells act to downregulate E-cadherin in cancer cells. The dose-dependent response in the

Figure 3
Flow cytometry data for the positive control and the experimental fraction. Brightness is on the x-axis and is on a log scale. The CM-treated cells showed several times less fluorescence than the untreated cells.

Figure 4
The effect of 184A1 treatment on cell binding. The CM-treated cells (right) showed a marked decrease in percent binding as compared to the untreated cells (left) (p < 0.005).
Finally, there are inherent limitations involved in using CM since it is unclear exactly what secreted product of the normal cells is responsible for the motility and E-cadherin downregulation. However, preliminary work in our lab suggests that the active factor in the CM is laminin-5, a basement membrane extracellular matrix macromolecule shown to play a role in both motility induction and adhesion (Giannelli et al., 1997; Goldfinger et al., 1998; Quaranta and Giannelli, 2003). A future goal will be to test laminin-5 and other factors suspected to affect motility and adhesion.

Conclusion

The connection between loss of adhesive molecules and metastasis is strongly supported in scientific literature. However, it was unclear whether motility factors acted by affecting adhesion molecules. Since normal breast cells occur in the same microenvironment as breast cancer cells and can sometimes only be a cell’s distance apart, how their secretions affect cancer cells becomes extremely significant. This study helps to elucidate the mechanism by which motility factors produced by the microenvironment of breast cancer cells can induce motility and shows that indeed one of the effects of treating cancer cells with CM is a decrease in E-cadherin levels. Though motility factors probably act through multiple pathways, uncovering each one is a significant step to developing treatments that can inhibit their motility-promoting effects.

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Works Cited


