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RESEARCH ARTICLE

Dual Roles of E-Cadherin in Prostate Cancer Invasion

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Abstract The role(s) of E-cadherin in tumor progression, invasion, and metastasis remains somewhat enigmatic. In order to investigate various aspects of E-cadherin biological activity, particularly in prostate cancer progression, our laboratory cloned unique subpopulations of the heterogeneous DU145 human prostatic carcinoma cell line and characterized their distinct biological functions. The data revealed that the highly invasive, fibroblastic-like subpopulation of DU145 cells (designated DU145-F) expressed less than 0.1-fold of E-cadherin protein when compared to the parental DU145 or the poorly invasive DU145 cells (designated DU145-E). Experimental disruption of E-cadherin function stimulated migration and invasion of DU145-E and other E-cadherin-positive prostate cancer cell lines, but did not affect the fibroblastic-like DU145-F subpopulation. Within the medium of parental DU145 cells, the presence of an 80 kDa E-cadherin fragment was detected. Subsequent functional analyses revealed the stimulatory effect of this fragment on the migratory and invasive capability of E-cadherin-positive cells. These results suggest that E-cadherin plays an important role in regulating the invasive potential of prostate cancer cells through an unique paracrine mechanism. J. Cell. Biochem. 91: 649–661, 2004.

Key words: E-cadherin; prostate cancer; invasion

Prostate cancer is a leading cause of cancer-related deaths among men in the US, with an estimated 28,900 deaths in 2003 [American Cancer Society, 2003]. Despite the enormity of these statistics, prostate cancer remains a relatively understudied disease with respect to its biology and molecular mechanisms of action.

The majority of prostate cancer cases arise from acquired somatic mutations associated with aging, whereas only 10% may be due to familial predisposition. Molecular genetic studies have identified many chromosomal aberrations and candidate mutated genes, in correlation with different stages of prostate cancer development and progression. The actual sequence of these mutations is currently unknown and in all probability may not conform to simple linear progression models [Ichikawa et al., 1991; Kallioniemi and Visakorpi, 1996; Abate-Shen and Shen, 2000; Dong, 2001]. One of the most important genetic changes takes place on the long arm of chromosome 16. Although deletion of 16q22 has been detected in only a few cases by cytogenetic analysis [Webb et al., 1996; Zitzelsberger et al., 1996], the frequency is as high as 31% (105 of 339 cases) in CGH studies—with most cases involving 16q13-23 [Dong, 2001].

Abbreviations used: sE-CAD, soluble E-cadherin fragment; rhE-cad/Fc, recombinant human E-cadherin/Fc chimeric protein; MICS, membrane invasion culture system; Mega-MICS, a large-scaled MICS; UEC, primary culture of urethral epithelial cells; CM, conditioned medium.

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In one study, deletion of 16q occurred in 11 (55%) of the 20 metastases examined [Cher et al., 1996]. A strong candidate gene for this region is E-cadherin gene, which is mapped to 16q22.1 [Natt et al., 1989]. In addition, expression of E-cadherin has been found to be reduced or absent in high-grade prostate cancers [Umbas et al., 1992].

The majority of diagnosed prostatic tumors remain localized and rarely produce dramatic clinical symptoms, while a subset of these tumors (roughly one in three) progresses to life-threatening malignancies [Abate-Shen and Shen, 2000]. In fact, 5 year survival rates for patients diagnosed with prostate cancer decrease dramatically, from 98.6% when the tumors were localized, to 29.8% after the tumor cells have spread to distant sites within the body (e.g., bone, liver, and brain). Therefore, an in-depth understanding of the mechanisms underlying the process of invasion and metastasis is a central goal of prostate cancer research.

Invasion and metastasis are acquired properties during tumor progression, and involve cancer cells losing intercellular contact, becoming motile, and invading surrounding tissues. A three-step theory of tumor cell invasion, which describes the initial process of the complex metastatic cascade was originally proposed by Liotta et al. [reviewed in Liotta et al., 1986]. The initial step involves the attachment of tumor cells via cell surface receptors to components of the extracellular matrix (ECM). The second step is defined by a digestion of the ECM by cell secreted proteolytic enzymes. The third step consists of migration of tumor cells into the region of matrix modified by proteolysis. However, recent studies have provided additional molecular clues indicating the dynamic and complex interactions that occur between tumor cells and their microenvironment during these steps [reviewed in Liotta and Kohn, 2001; Davies et al., 2001].

Previous reports have indicated that E-cadherin is a strong suppressor of invasion in various systems [Vleminckx et al., 1991; Berx et al., 1995; Miyaki et al., 1995; Luo et al., 1997, 1999; Furuyama et al., 2000]. However, precisely how E-cadherin mediates this suppressive function remains poorly understood. Although some studies have suggested an inhibitory role of E-cadherin on the transactivational activity of β-catenin [Sadot et al., 1998; Orsulic et al., 1999], this scenario is yet to be demonstrated in prostate cancer. E-cadherin is a 120 kDa transmembrane glycoprotein. It is a member of the cadherin family of calcium-dependent cell adhesion molecules, which binds to one another in a homophilic manner and is involved in selective cell–cell recognition and adhesion [Takeichi, 1990]. The cytoplasmic domain of E-cadherin interacts with catenins and cytoskeletal proteins to maintain the stability of cell–cell adhesion complexes and, ultimately, tissue integrity [Ozawa et al., 1989; Aberle et al., 1994]. The extracellular portion of E-cadherin consists of five tandemly repeated domains, which are essential for Ca^{2+} binding and homophilic interaction. Results from nuclear magnetic resonance spectroscopy analysis and X-ray crystallography studies have suggested that two E-cadherin extracellular domain monomers align in parallel to form dimers [Overduin et al., 1995; Shapiro et al., 1995]. E-cadherin dimers from the interacting cell membranes of opposing cells then interact like a "linear-zipper" at the site of intercellular contact [Shapiro et al., 1995]. Proteolytic cleavage of the extracellular domains of E-cadherin yields a secreted, soluble fragment (sE-CAD), which has an apparent molecular weight of 80 kDa [Damsky et al., 1983], and can disrupt cell-cell adhesion [Wheelock et al., 1987]. Of special note are recent studies demonstrating a clinical potential for the role of circulating soluble E-cadherin fragment as a diagnostic tumor marker [Katayama et al., 1994; Gofuku et al., 1998].

In the current study, we asked whether E-cadherin is involved in regulating invasion in human prostate cancer. In order to answer this question, we took advantage of a large-scale in vitro invasion chamber developed in our laboratory, known as MegaMICS (a large-scaled membrane invasion culture system (MICS)), to select and enrich the highly and poorly invasive subpopulations from the human prostate cancer cell line DU145. We then characterized the two subpopulations, especially with respect to their E-cadherin status. Here, we present evidence that E-cadherin is important for maintaining the poorly invasive, epithelial-like phenotype. Additional data indicate that an 80 kDa E-cadherin fragment is secreted into the conditioned medium (CM) of cultured DU145 cells. Further characterization revealed the chemotactic and invasion-promoting properties of this soluble E-cadherin fragment.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The human prostate cancer cell lines DU145 and LNCaP were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The MLC8891 immortalized human prostate epithelial cell line was a kind gift from the laboratory of Dr. Ruth Sager and Dr. Arthur Pardee of Dana-Farber Cancer Institute, and was maintained in a serum-free keratinocyte growth medium supplemented with bovine pituitary extract (Invitrogen, Carlsbad, CA). The primary culture of urethral epithelial cells (UEC) was a kind gift from Dr. Michael Apicella (University of Iowa) and was maintained in the prostate epithelial cell basal medium (PrEBM) supplemented with BEGM Singlequots (Clonetics, San Diego, CA) and 0.1% gentamycin sulphate (Gemini Bioproducts, Woodland, CA). Unless specified otherwise, all other cell lines were maintained in DMEM medium (Invitrogen) supplemented with 10% FBS and 0.1% gentamycin sulphate (Gemini Bioproducts). All cell cultures were maintained at 37°C, in a humidified environment with 5% CO_2. Culture medium was renewed every 2–3 days. All cell lines were tested regularly for *Mycoplasma* contamination using a PCR-based detection system (Roche, Indianapolis, IN).

Selection of Highly Invasive and Poorly Invasive Subpopulations of DU145 Cells

DU145 cells were selected based on their in vitro invasive ability through the reconstituted basement membrane assembled within the MegaMICS chambers as described previously [Seftor et al., 1990]. Briefly, cells at approximately 85% confluence were removed from the tissue culture flask by incubating for 5 min in 2 mM EDTA in PBS. These cells were then centrifuged and resuspended in DMEM medium containing 1× $MITO^+$ serum supplement (Discovery Labware, Bedford, MA) and gentamycin sulphate (Gemini Bioproducts). The cell suspension (3×10^6 cells/well) were seeded on the top well of the MegaMICS chambers, which is separated from the lower wells by a 10 μm-pore polycarbonate membrane (Osmonics, Livermore, CA) coated with a uniform thickness of defined basement membrane matrix (human laminin, collagen IV, and gelatin; Sigma, St. Louis, MO). Cells that invaded through the coated membrane over a 24 h period were collected, cultured, and reselected two more times. Cells that remained on top of the membrane were also collected, recultured, and subjected to two more rounds of selection for non-invasiveness. The enriched invasive cells were designated DU145-F, whereas the enriched non-invading cells were designated DU145-E.

Electron Microscopy

Cultures were grown to near confluence in 12-well tissue culture dishes (Corning Incorporated, Corning, NY) for transmission electron microscopy (TEM), and in 12-well tissue culture dishes on glass coverslips for scanning electron microscopy (SEM). Monolayer cultures were fixed in cold 2.5% glutaraldehyde in 0.2M cacodylate buffer (pH 7.2) for 1 h, followed by a cacodylate buffer rinse prior to post-fixation in 1% osmium tetroxide in cacodylate buffer. TEM specimens, together with culture dish substrate, were polymer embedded by routine means, and thin sections were cut and mounted on slotted copper grids coated with celloidin. Sequential staining with uranyl acetate and lead citrate preceded examination under a Hitachi H7000 electron microscope. Specimens for SEM on glass coverslips were critical-point dried from absolute alcohol, mounted on copper stubs and coated with gold–palladium prior to examination under a Hitachi S4000 scanning electron microscope.

Cell Lysate and Western Blot Analysis

Cells at near confluence (approximately 85%) from a T-75 tissue culture flask were washed twice with ice-cold PBS and lysed in 1 ml RIPA buffer (100 mM Tris, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS) containing 2 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 mM sodium vanadate (Sigma) for 5 min at 4°C. The lysate was then collected by cell scraping, sonicated for 2–3 s, and centrifuged at 14,000*g* for 30 min at 4°C. The protein concentration was determined by using the micro BCA assay kit (Pierce, Rockford, IL). For Western blot analysis of the full-length E-cadherin, 20 μg of total protein was subjected to 10% SDS–PAGE under reducing conditions, and the protein bands were transferred onto

Immobilon-P membrane (PVDF; Millipore Corporation, Bedford, MA). The membrane was blocked overnight at 4°C in blocking buffer (6% non-fat dry milk in 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween-20) and incubated for 1 h at room temperature in anti-E-cadherin Ab (BD Transduction Laboratories, Lexington, KY) diluted 1:2,500 in blocking buffer. After 30 min of extensive washes, the blot was incubated for 1 h at room temperature in HRP-conjugated goat-anti-mouse 2° Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:10,000 in blocking buffer, followed by 30 min of extensive washes before being developed with an ECL Western blot detection kit (Perkin-Elmer Life Sciences, Boston, MA). Western blot analyses for β-catenin, p120cas, cytokeratin 18, vimentin, and actin were performed similarly, except that the 1° antibodies were from different sources: β-catenin and p120cas (BD Transduction Laboratories), cytokeratin 18 and actin (Chemicon International, Temecula, CA), and Vimentin (clone V9; Sigma).

Invasion Assay and Chemomigration Assay

Tumor cells (5×10^4) were seeded into the upper wells of the analytical MICS chambers [Hendrix et al., 1987], which are separated from the lower wells by a 10 μm-pored polycarbonate membrane (Osmonics) coated with a uniformed thickness of defined human basement membrane matrix proteins. Where indicated, 2.0 μg/ml final concentration of anti-E-cadherin Ab (clone HECD-1; Zymed Laboratories, South San Francisco, CA; a blocking antibody) or an isotype-control Ab was added to the upper wells. After 24 h of incubation at 37°C, cells that invaded through the membrane were collected, stained, and counted. The relative invasive potential was calculated by comparing the number of invading cells plus or minus the anti-E-cadherin Ab. Chemomigration assay was performed in a similar way, except that the polycarbonate membrane was soaked in a very diluted solution of gelatin (0.1 mg/ml in 0.02 M acetic acid) for 1 h at room temperature and the 1.0 μg/ml final concentration of chemoattractant (rhE-cad/Fc) or rabbit-anti-mouse IgG (control) was included in the lower wells. Cells were allowed to migrate across the polycarbonate membrane for 6 h before being harvested, stained, and scored as previously described [Hendrix et al., 1987].

Conditioned Medium (CM)

Cells at approximately 85% confluence were washed twice with serum-free DMEM medium and allowed to grow in DMEM medium containing 1× MITO$^+$ serum supplement and gentamycin sulphate. The CM was collected 48 h later. Proteinase inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM PMSF, and 2 mM sodium vanadate) were added immediately. The CM was then centrifuged to remove cellular debris, and filtered through a 0.22 μm syringe-driven filter unit (Millipore Corporation) before use.

Detection of E-Cadherin Fragment in the CM

In order to detect the presence of 80 kDa sE-CAD in the CM collected from cells in culture, 50 ml of CM was concentrated to 1 ml by centrifuging through the YM-30 Centrifugal Filter Device (Amicon Bioseparations, Beverly, MA). The concentrated sample was then immunoprecipitated by incubating with 5 μg of anti-E-cadherin Ab (clone HECD-1; Zymed) or an isotype-control Ab (anti-FAK Ab) in a rotating device for 1 h, at 4°C. The mixture was then incubated for another hour with the addition of a rabbit-anti-mouse 2° Ab (Jackson ImmunoResearch Laboratories). Protein A sepharose beads (60 μg; Sigma) was added and the mixture incubated for an additional 1 h at 4°C. The immunocomplex was collected by centrifugation, washed twice with ice-cold PBS, and boiled for 5 min in sample buffer before being subjected to Western blot analysis.

Substrate Incorporated SDS–Polyacrylamide Gel Electrophoresis (Zymography)

Relative, extracellular levels of MMP-9 and MMP-2 were determined using zymography as previously described [Seftor et al., 2002]. After plating 1×10^6 cells into the tissue culture flasks for 1 h in complete media, the media was removed and replaced with serum-free medium containing mito+. After 48 h, the medium was removed, microfuged at 2,000 rpm to remove cell debris and the supernatant mixed two parts to one part Laemmli sample buffer without reductant. Without boiling, equal volumes per sample were loaded onto a 10% SDS–polyacrylamide gel and the gel run and processed as previously described. Three different zymograms from three replicate experiments yielded results with values within 5% of the values shown for the zymogram in Figure 2C.

RESULTS

Morphological Characterization of Prostatic Cancer Subpopulations

After three rounds of selection through basement membrane-coated membranes, two cell subpopulations with distinct morphology were expanded from a heterogeneous population of DU145 human prostate cancer cells. Consistently, parental DU145 cultures displayed the mixture of elongated and polygonal cells (Fig. 1A). Poorly invasive cells obtained from the top wells of the large-scaled invasion chambers (MegaMICS) displayed epithelial-like morphology, and hence were designated DU145-E (Fig. 1B). In contrast, the majority of invasive cells collected from the lower wells of the MegaMICS consisted of elongated, fibroblastic-like morphology, and therefore were designated DU145-F (Fig. 1C).

SEM revealed that DU145-E cells exhibited a polygonal shape and possessed many filopodial extensions from the cell surface contacting adjacent cells (Fig. 1D). The cell surface exhibited many prominent cytoplasmic plicae and long microvillous projections, many of which were concentrated over the nuclear region of the cell. In contrast, SEM of DU145-F demonstrated the generally more elongated nature of most cells in culture, together with a paucity of cytoplasmic extensions projecting to adjacent cell surfaces (Fig. 1F, compared to 1D). Filopodia, however, appeared to extend from the leading edges of migrating cells. In addition, DU145-F cells typically exhibited membranous folds and short microvilli on their cell surfaces. Upon examination under the transmission electron microscope (TEM), DU145-E cells were found to contain numerous, long projections on the cell surface and displayed distinct junctional complexes between overlapping cells (Fig. 1E, arrows). The cytoplasm of these cells contained abundant glycogen and profiles of rough endoplasmic reticulum (RER). In contrast, TEM of DU145-F cells (Fig. 1G) demonstrated short surface projections and a loose association of overlapping cells, lacking junctional regions. These cells were typified by bizarre-shaped nuclei with complex nucleoli, abundant mitochondria, numerous cytoplasmic dense bodies, as well as profiles of RER. These data demonstrate distinctive morphological characteristics associated with the selected subpopulations of prostatic cancer cells isolated from the parental heterogeneous DU145 cell line.

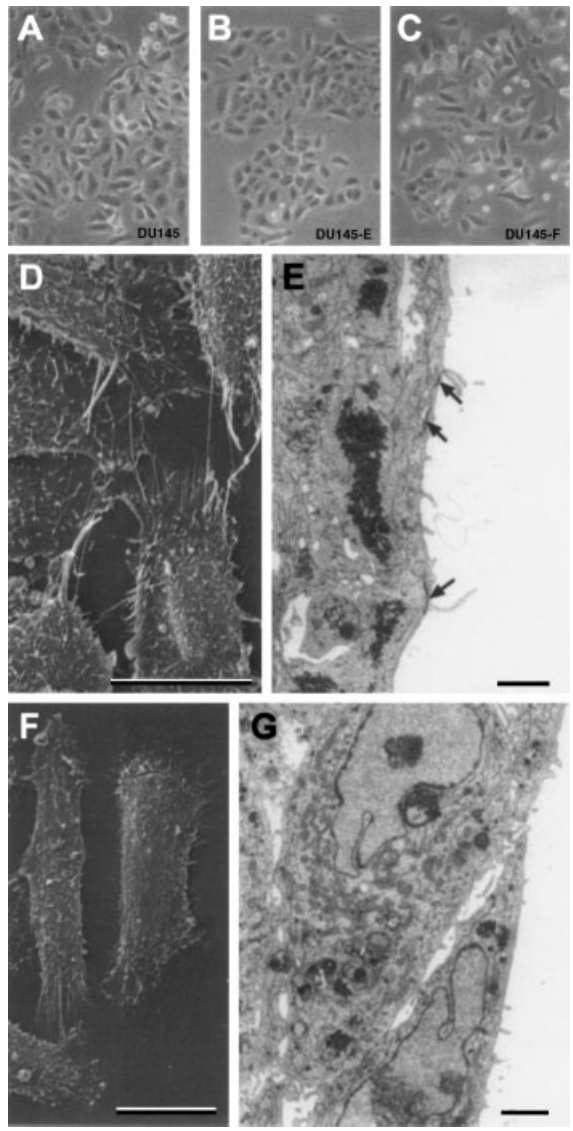

Fig. 1. Morphological analyses of human prostatic cancer cells. **A**, **B**, and **C** are phase contrast micrographs of parental DU145 cells, and DU145-E and DU145-F subpopulations, respectively. Note the mixture of elongated and polygonal cells in the parent culture; (A) the more epithelial growth pattern typical of DU145-E cultures; (B) and the predominance of cells with an elongated, fibroblastic-like phenotype typical of DU145-F cultures (C). **D** and **E** show micrographs from scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of DU145-E cells, respectively. Arrows indicate junctional regions between overlapping cells. **F** and **G** are SEM and TEM of DU145-F cells, respectively. (Bars in D and F are 25 μm. Bars in E and G are 2 μm.)

Protein Expression and Invasion Profiles

Previous studies have implicated E-cadherin as an important regulator of cancer invasion

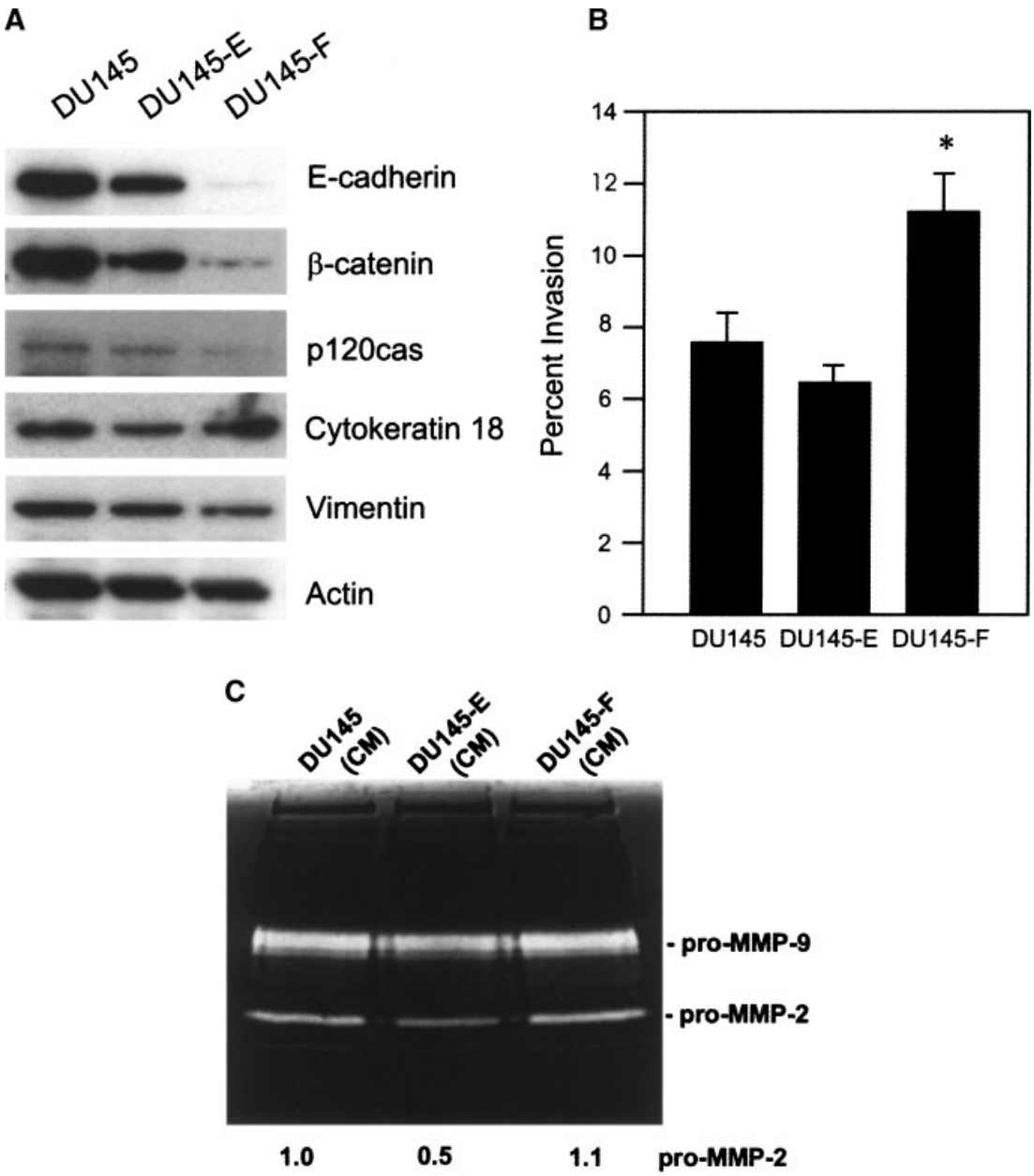

Fig. 2. Western blot analyses, in vitro invasive ability, and zymography of DU145, DU145-E, and DU145-F samples. **A**: Note the difference in E-cadherin, β-catenin, and p120cas protein expression between the clones. No significant differences were noted in the protein levels of cytokeratin 18 or vimentin. Actin served as an equal loading control. **B**: Asterisk (*) over the bar of invasive ability indicates a statistically significant difference (compared to that of DU145; $P < 0.05$ based on paired *t*-test). **C**: Representative zymogram (one of three from three differently prepared set of samples) of serum-free, conditioned media (CM) from DU145, DU145-E, and DU145-F cells grown on plastic for 48 h. Relative levels of pro-MMP-2 were compared to a normalized value of 1.0 for the parental DU145 cells by digitizing the zymogram and analyzing the areas corresponding to pro-MMP-2 using ScionImage 4.0.2β software.

in prostatic and other types of carcinomas [Frixen et al., 1991; Vleminckx et al., 1991; Miyaki et al., 1995; Luo et al., 1999]. To determine whether this is also true in our model system, Western blot analyses of E-cadherin and other select associated proteins were conducted (Fig. 2A). The data indicated that DU145-E cells expressed a similar amount of E-cadherin protein when compared to the parent DU145 cells. However, DU145-F cells expressed a nearly undetectable level of E-cadherin protein. The levels of two E-cadherin-

associated proteins, β-catenin and p120cas, appeared to correlate with the level of E-cadherin, whereas the protein levels of cytokeratin 18 (an epithelial marker) and vimentin (a mesenchymal marker) were similar across the panel, indicating the acquisition of an interconverted phenotype.

To compare the in vitro invasiveness of the selected subpopulations with that of the parental cell line, detached cells were subjected to analytical MICS chambers as described in "Materials and Methods." As shown in Figure 2B, the invasiveness of DU145, DU145-E, and DU145-F cells were $7.58 \pm 0.85\%$ (SD), $6.47 \pm 0.45\%$ (SD), and $11.22 \pm 1.05\%$ (SD), respectively. Zymographic analysis of MMP-2 and MMP-9 activity within the CM of the parental DU145 and selected subpopulations revealed approximately twice the amount of pro-MMP-2 secreted by the parental and highly invasive DU145-F cells compared to the Du145-E cells (Fig. 2C). These data show the acquisition of a more aggressive phenotype by the DU145-F cells in association with low expression levels of E-cadherin, β-catenin, and p120cas, an increase in their invasive ability and higher extracellular levels of pro-MMP-2.

Experimental Disruption of E-Cadherin Function

In order to test whether interfering with E-cadherin function would result in increased cellular migration and invasion, we utilized the commercially available recombinant human E-cadherin/Fc chimeric protein (rhE-cad/Fc) and the E-cadherin blocking Ab (HECD-1). As shown in Figure 3A, the migration rate of E-cadherin-positive cells (UEC, LNCaP, MLC8891, DU145, and DU145-E) increased significantly in response to 1.0 μg/ml (final concentration) of rhE-cad/Fc in the lower wells of the MICS chambers. However, the migration rate of DU145-F cells, which expressed very little to no E-cadherin protein, did not increase in response to the same stimulus. Results from a concurrent set of experiments using E-cadherin blocking Ab (Fig. 3B) indicated that the invasion rate of MLC8891 and DU145-E was increased by almost twofold in response to the addition of 2 μg/ml (final concentration) of the E-cadherin blocking Ab in the upper wells of the MICS chambers. Interestingly, the parental DU145 cells (which are heterogeneous in E-cadherin protein expression) and the invasive DU145-F cells did not respond to the blocking Ab treatment.

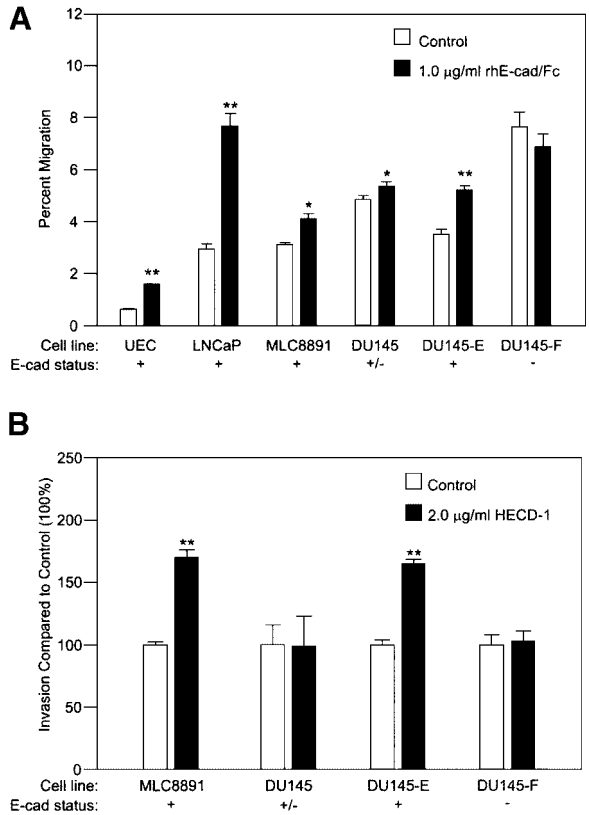

Fig. 3. Migration (**A**) and relative invasiveness (**B**) of human prostate cancer cell lines (LNCaP, DU145), sublines (DU145-E and DU145-F), immortalized prostatic epithelial cells (MLC8891), and cells from primary culture of urethral epithelium (UEC) in response to treatment with the recombinant human E-cadherin-immunoglobulin Fc chimeric protein (rhE-cad/Fc) and E-cadherin blocking Ab (HECD-1). A single asterisk (*) indicates the statistically significant difference with P value < 0.05 based on paired t-test. Double asterisks (**) indicate the statistically significant difference with P value < 0.01 based on paired t-test. The invasion rates of untreated cells (B) are normalized to 100% and those of treated cells are expressed as percentage compared to control. DU145-F cells were assigned negative status for E-cadherin protein expression because we could detect the E-cadherin band only when the film was overexposed during Western blot analysis. Unselected DU145 cells, although positive for E-cadherin, were assigned ± status due to the fact that they consist of both DU145-E and DU145-F selected cells.

Detection and Functional Analyses of an E-Cadherin Fragment in CM

It has been shown that proteolytic cleavage of E-cadherin can lead to the release of an 80 kDa E-cadherin extracellular fragment which has an invasion-promoting effect on epithelial cancer cells [Damsky et al., 1983; Noe et al., 2001]. To test whether the 80 kDa E-cadherin fragment

was present in the cultured medium of DU145 prostate cancer cells, we collected the CM and performed an immunoprecipitation as described in the "Materials and Methods." Subsequent Western blot analyses indicated the presence of an 80 kDa protein which was recognized by the anti-E-cadherin Ab, but not by the isotype-control Ab (Fig. 4A).

To test whether the CM collected from cultured DU145 cells exerted an invasion-promoting effect, we performed an in vitro invasion assay, with or without 25× concentrated DU145 CM added in the lower wells of the invasion chambers. As shown in Figure 4B, the invasion rates of LNCaP prostate cancer cells increased approximately 2.5-fold compared to control ($2.37 \pm 0.07\%$ SD vs. $0.87 \pm 0.06\%$ SD). A similar result was observed with the MLC8891 immortalized prostate epithelial cells ($3.73 \pm 0.06\%$ SD vs. $1.56 \pm 0.22\%$ SD). Due to the low concentration of the 80 kDa E-cadherin fragment in the CM isolation, use of the purified fragment was technically difficult. Therefore, we employed a

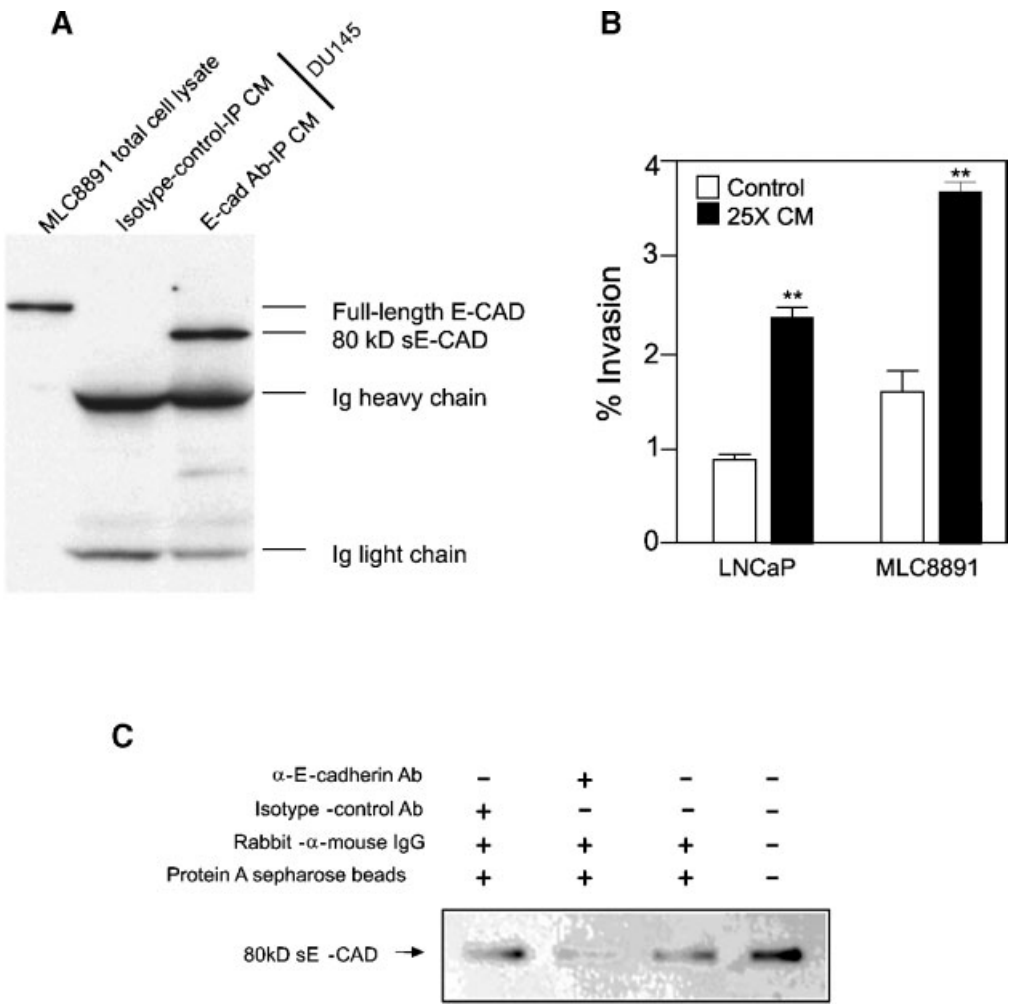

Fig. 4. Immunoprecipitation, Western blot analysis, and in vitro invasive ability related to the CM of DU145 cells. Immunoprecipitation and Western blot analysis revealed an 80 kDa E-cadherin fragment in the CM of DU145 cells (**A**). The DU145 CM was concentrated 50-fold and subjected to immunoprecipitation using either an anti-E-cadherin or an IgG isotype (control) antibody. A total cell lysate from an immortalized prostate epithelial cell line, ML8891, was used as a positive control for E-cadherin. **B**: Effect of DU145 CM on the invasive potential of ML8891 and LNCaP prostate cancer cells. A 25-fold concentrate of DU145 CM was added to the samples and changes in the cells' invasive potential compared to the untreated control cells. The double asterisks (**) indicate statistically significant difference in invasiveness ($P < 0.01$, paired *t*-test). **C**: Western blot analysis demonstrating the level of sE-CAD that remains in the CM after various immunoprecipitation conditions.

subtractive strategy in which immunoprecipitation with E-cadherin specific Ab was used to remove the 80 kDa E-cadherin fragment from the CM (Fig. 4C). Subsequent migration assays indicated that the untreated CM stimulated cellular migration by 1.3 to 2.8-fold, whereas the E-cadherin-stripped CM yielded similar results to those of the basal medium treatment (Table I), although the stripping of the 80 kDa E-cadherin fragment did not appear to be complete. Thus, this experimental approach demonstrated that removal (or at least partial removal) of secreted, proteolytically cleaved E-cadherin from the CM resulted in a reversal of the stimulated migratory activity.

DISCUSSION

The objective of this study was to investigate the importance of E-cadherin in a human prostate cancer model system. Specifically, we asked whether E-cadherin is involved in regulating invasion in human prostate cancer cell lines. We selected two subpopulations of DU145 cells on the basis of their in vitro invasive ability and subsequently characterized them, with the main focus on E-cadherin status. We found that the highly invasive subpopulation of DU145 cells (designated DU145-F) expressed less than one-tenth the E-cadherin protein when compared to the parental DU145 cells and/or the poorly invasive DU145 subpopulation (designated DU145-E). Experimental treatments known to disrupt E-cadherin function induced migration and invasion of DU145-E and other E-cadherin-positive prostate cancer and urethral epithelial cell lines, but did not affect DU145-F. We were also able to detect the presence of an 80 kDa E-cadherin fragment in the medium collected from cultured DU145 cells. Subsequent tests revealed the stimulating effect of this fragment on invasion and migration. These results offer a new human model in which to study the role E-cadherin plays in regulating invasion of prostate cancer cells.

Our data indicated that the DU145 human prostate cancer cell line is not a homogeneous line, at least with respect to E-cadherin protein expression. This finding is consistent with the currently accepted view that prostate cancer is heterogeneous and multiclonal in nature—features that are closely linked to genetic instability. Consistent with these findings, reduced expression of E-cadherin has been reported in clinical samples of prostate cancers and was found to be correlated with poor prognosis [Umbas et al., 1992, 1994]. Along the same line, the adhesive function of E-cadherin was found to be compromised in the highly invasive human prostate cancer cell line PC-3, by a deletional mutation in the gene encoding an associated protein α-catenin [Morton et al., 1993]. Deletion of chromosome 5q which harbors the α-catenin gene is a frequent event in prostate cancer progression [McPerson et al., 1994; Abate-Shen and Shen, 2000]. From a functional perspective, our data indicated that E-cadherin may be involved in regulating the invasion of human prostate cancer cells. The DU145-F subline, which expresses a very low to nondetectable level of E-cadherin protein, was highly invasive when compared to DU145-E and parental DU145 cells which express at least 10-fold more E-cadherin protein (Fig. 2). This finding is consistent with reports by Luo et al. [1997, 1999] which demonstrated in Dunning rat tumor clonal cell lines that E-cadherin functioned as a suppressor of invasion. Therefore, evidence from our model system suggested that the loss of E-cadherin expression may contribute to the aggressive transformation and poor prognosis by rendering the cells more invasive. Curiously, throughout the selection

TABLE I. Relative Migration of Human Prostate Cancer Cell Lines and Sublines In Vitro in Response to Various Stimuli

[a]Stimuli in lower chamber	[b]Relative migration compared to control (100%)			
	LNCaP	DU145	DU145-E	DU145-F
Basal medium	100 ± 12% SE	100 ± 8% SE	100 ± 17% SE	100 ± 4% SE
Untreated CM	282 ± 38% SE	179 ± 3% SE	207 ± 35% SE	130 ± 10% SE
E-cad Ab-stripped CM	101 ± 4% SE	94 ± 9% SE	121 ± 19% SE	111 ± 4% SE

[a]Basal medium served as a negative control.
[b]Migration of cells treated with basal medium was normalized to 100%.

process, the DU145-E and DU145-F cells co-expressed cytokeratin 18 (an epithelial intermediate filament marker) and vimentin (a mesenchymal intermediate filament marker), suggesting their unique ability to maintain an interconverted phenotype throughout the selection process.

Morphological studies support the heterogeneity of the DU145 tumor cell line as well. The isolation of tumor sublines differing both in morphology and in invasive potential is consistent with earlier findings in another prostate tumor, the Dunning rat adenocarcinoma [Feuchter et al., 1980; Thompson et al., 1985; Luo et al., 1997; Sharma et al., 2002]. Both systems exhibit a heterogeneous parental tumor line, from which less invasive, epithelioid sublines and fibroblastic, more highly invasive sublines, have been isolated. Invasive potential in both systems is E-cadherin-linked; the epithelioid, less invasive cells express E-cadherin, whereas the more invasive fibroblastic sublines have greatly reduced or absent E-cadherin expression. The morphologic phenotypes across species bear remarkable similarities. Polygonal, epithelial-like cells, as assessed by SEM, extend numerous, long filopodial processes in culture that contact adjacent cells. The cells tend to aggregate with sheet-like and filopodial processes in contact. These contacting regions are the sites of formation of desmosomal junctions. By contrast, the fibroblastic sublines extend processes of the cell membrane predominantly at the leading edge of elongate, motile cells, and close clustering of cells exhibiting cellular junctions was not observed in the present study. By TEM, the cytoplasm of the DU145 epithelioid cells exhibited large stores of cytoplasmic glycogen, and abundant rough endoplasmic reticulum, features frequently observed in the well-differentiated Dunning tumor [Feuchter et al., 1980]. In the current study, fibroblastic DU145 cells exhibited bizarre nuclei (reminiscent of the Dunning tumor), numerous mitochondria, lipid inclusions, and cytoplasmic dense bodies. These similarities in morphology serve as additional criteria suggesting that both rat and human adenocarcinoma models undergo similar phenotypic transformation, and add to the body of evidence suggesting that the Dunning tumor is a suitable animal model for human prostatic adenocarcinoma.

The importance of E-cadherin in suppressing cellular invasion and migration was confirmed in this study. Treatments that are known to disrupt E-cadherin function induced the invasion and/or migration of E-cadherin-positive cells, but not that of DU145-F which expressed a very low level of E-cadherin protein. This suggested that the observed stimulating effects on invasion and migration were the result of E-cadherin's functional disruption (Fig. 3). This finding supported our hypothesis that E-cadherin is important for maintaining the normal, non-invasive, phenotype and that disruption of E-cadherin function results in an increase in migration and invasion. In light of this evidence, it is logical to conclude that any agent (either synthetic or biological) that has the ability to disrupt the adhesive function of E-cadherin and/or other cell adhesion molecules may contribute to the migration and invasion of prostate cancer cells and, therefore, should be avoided as a therapeutic approach. By the same logic, agents that promote E-cadherin expression and adhesive function would have promising therapeutic potential for treatment and/or prevention of prostate cancer.

The fact that we were able to detect an 80 kDa fragment of E-cadherin in the CM of cultured prostate cancer cells, using an antibody specific to the extracellular domain of E-cadherin, suggests that proteolytic cleavage of E-cadherin extracellular portion took place, at least in our experimental model. A recent study [Noe et al., 2001] demonstrated that MMP-3 and MMP-7 may be responsible for the cleavage of E-cadherin extracellular domains in MCF-7 and MDCKts.src12 cells. In our study, the 80 kDa proteolytic fragments, which were released into the culture medium were shown to have an invasion-promoting effect, the highest levels of MMP activity, particularly pro-MMP-2, were associated with the parental DU145 cells and the highly invasive DU145-F subpopulation. However, the potential role of MMP-2 in E-cadherin proteolytic cleavage is unclear at this time. From a translational perspective, our in vitro data confirm a recent clinical report revealing that the ratio of Gleason score:MMP (MMP-2 and MMP-9):E-cadherin provides the strongest association with pathologic stage compared with other predictors [Kuniyasu et al., 2003]. The data also demonstrated that the 80 kDa E-cadherin fragment can display chemotactic properties (Table I). The fact that the E-cadherin-stripped CM showed decreased induction of migration, even when the removal

of sE-CAD was not complete, indicates that a critical threshold concentration of sE-CAD is required for its ability to act as a chemoattractant. Taken together, these data demonstrate the complex nature of E-cadherin involvement in the regulation of invasion in which intact E-cadherin molecules function to prevent migration, whereas proteolytic fragments of E-cadherin function in the opposite manner.

Our hypothetical model, presented in Figure 5, demonstrates how E-cadherin may regulate the invasion of prostate cancer cells. Intact E-cadherin molecules would normally function to hold epithelial cells together and maintain tissue integrity, as well as prevent invasion and migration. The ability of E-cadherin to suppress invasion is regulated both temporally and spatially. Certain cellular or tissue changes which interfere with E-cadherin adhesive function may have different effects on invasion, depending on the time and location of fragment release. On the one hand, the extracellular fragment locally released into nearby epithelial tissue may function as a negative competitor of the full-length E-cadherin in establishing cell-cell adhesion, therefore allowing the cells to break away from their primary sites and enter into the stromal compartment, depending on the gradients of chemotactic factors (e.g., growth factors) and/or haptotactic factors (e.g., ECM molecules that are fixed in the connective tissue) that are present. On the other hand, a large amount of E-cadherin fragments would hypothetically be released into the lumenal side of the prostate glands. This could potentially, as our data indicated, function as a chemotactic

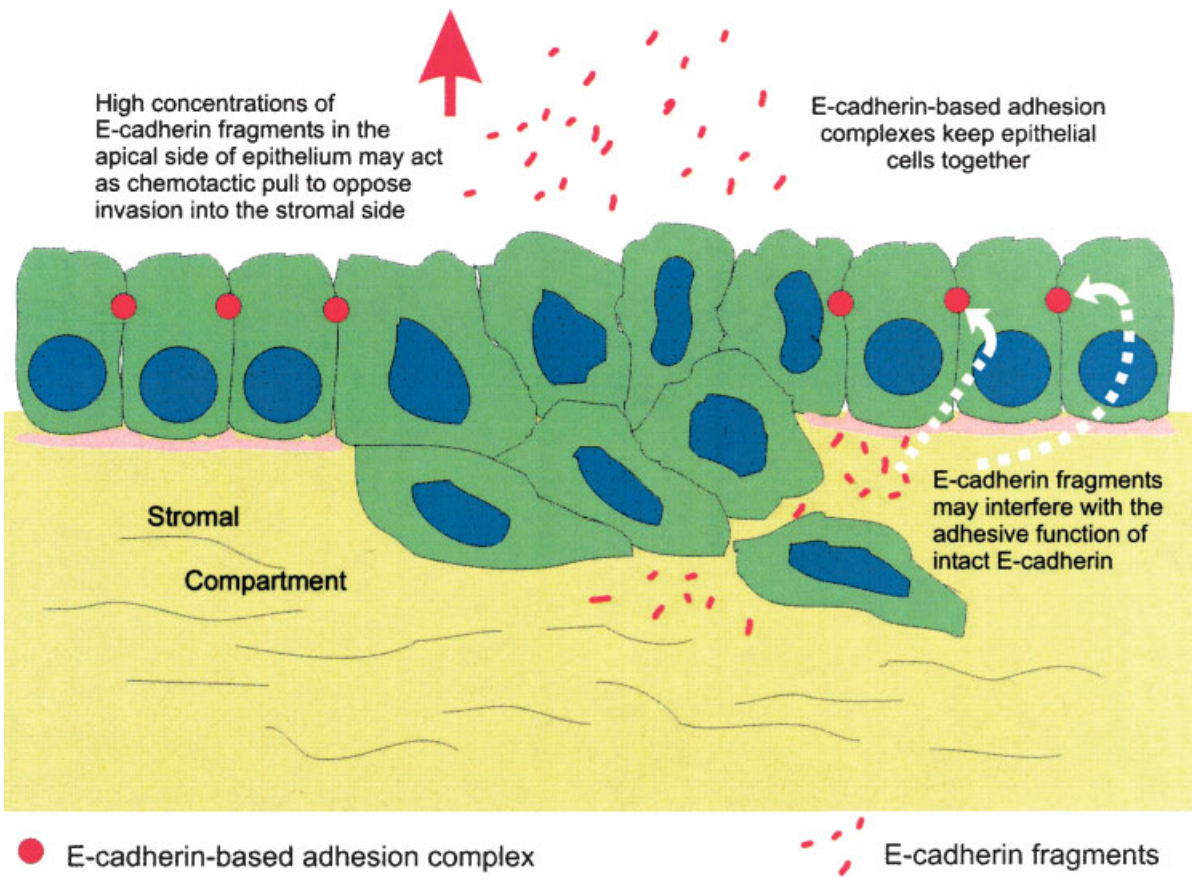

Fig. 5. A hypothetical model of how E-cadherin may regulate invasion. Full-length E-cadherin homodimers form adhesive junctions that function to maintain epithelial sheets and polarity, preventing the epithelial cells from becoming migratory. Proteolytic fragments of E-cadherin, however, may have different effects on invasion. First, the shredded fragments may function as a negative competitor that interferes with homophilic interaction of the full-length molecules, thus promoting migration and invasion. Under some conditions, a high concentration of the E-cadherin fragments released into the lumens of prostate glands may act as a chemotactic agent that induces cells to remain in the epithelial sheet, as opposed to invading into the stromal compartment.

agent attracting cells to migrate toward the lumenal side of the glands and thus oppose the direction of stromal invasion. This effect clearly would be dependent upon the minimum threshold concentration required for the fragment to function as a chemotactic factor. Nonetheless, the current study has illuminated the highly complex nature of the molecular underpinnings of E-cadherin in tumor progression, which will require additional scientific investigation.

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