Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1

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SUMMARY

The function of many transmembrane molecules can be altered by cleavage and subsequent release of their ectodomains. We have investigated ectodomain cleavage of the cell-cell adhesion and signal-transducing molecule E-cadherin. The E-cadherin ectodomain is constitutively shed from the surface of MCF-7 and MDCKts.srcC12 cells in culture. Release of the 80 kDa soluble E-cadherin fragment is stimulated by phorbol-12-myristate-13-acetate and is inhibited by overexpression of the tissue inhibitor of metalloproteinases-2. The metalloproteinases matrilysin and stromelysin-1 both cleave E-cadherin at the cell surface and release sE-CAD into the medium. The soluble E-cadherin fragment thus released inhibits E-cadherin functions in a paracrine way, as indicated by induction of invasion into collagen type I and inhibition of E-cadherin-dependent cell aggregation. Our results, therefore, suggest a novel mechanism by which metalloproteinases can influence invasion.

Key words: E-cadherin/catenin complex, Ectodomain shedding, Matrix metalloproteinase, Proteolysis, Invasion

INTRODUCTION

E-cadherin is a calcium-dependent cell-cell adhesion molecule which, together with the catenins interacting with its cytoplasmic tail, forms a powerful invasion suppressor complex. The activities of the E-cadherin/catenin complex are regulated at multiple levels including mutations of the E-cadherin gene (Berx et al., 1996), tyrosine phosphorylation of β-catenin through pp60src, c-Met or EGF receptor activation (Behrens et al., 1993; Shibamoto et al., 1994) and by disturbance of intracellular associations with catenins (Näthke et al., 1994; Vermeulen et al., 1995). Here we provide evidence that ectodomain fragments of E-cadherin may regulate E-cadherin functions in cells containing an intact E-cadherin/catenin complex.

Proteolytic shedding of the ectodomain of a variety of transmembrane proteins, including growth factor receptors and their ligands, cell-matrix and cell-cell adhesion molecules has been observed (Werb and Yan, 1998). In particular, ectodomain shedding has been described for cadherins. In the developing chick neural retina, proteolysis downregulates surface levels of neural cadherin (N-cadherin), resulting in the release of a soluble 90 kDa N-cadherin fragment. 1,10 phenanthroline inhibits N-cadherin shedding in retina organ culture, suggesting that a metalloproteinase is responsible for cleavage (Roark et al., 1992). The soluble 90 kDa N-cadherin fragment is an adhesive substrate and promotes attachment, spreading and neurite outgrowth of embryonic chick neural retina cells (Paradies and Grunwald, 1993). Metalloproteinase cleavage of vascular endothelial cadherin (VE-cadherin) releases a 90 kDa extracellular fragment and has been associated with the induction of apoptosis in endothelial cells (Herren et al., 1998).

E-cadherin ectodomain shedding was first suggested by the detection of a soluble 80 kDa fragment in the medium of the human breast cancer cell, MCF-7 (Wheelock et al., 1987). This E-cadherin fragment (sE-CAD) caused scattering of epithelial cells in culture, an effect that could be neutralized by antibodies against E-cadherin. E-cadherin cleavage and release of the 80 kDa sE-CAD also occurs during uterine invasion in pregnant mice and can be induced by treatment with oestradiol (Potter et al., 1996). sE-CAD activity was mimicked by synthetic E-cadherin peptides containing the histidine-alanine-valine amino acid sequence (Mbalaviele et al., 1995; Willems et al., 1995; Noë et al., 1999). This sequence is conserved in the family of classical cadherins and is important for homophilic binding (Overduin et al., 1995). E-cadherin functions were specifically inhibited in a paracrine way by E-cadherin peptides, as indicated by inhibition of aggregation, disturbance of epithelial morphology and induction of invasion (Noë et al., 1999).

The long-standing association of matrix metalloproteinase (MMP) activity and tumor cell invasion has been interpreted to be solely a result of degradation of extracellular matrix, a natural barrier to tumor invasion. However, the list of transmembrane targets of MMP proteolysis has grown and recently it has been suggested that the MMP stromelysin-1 leads directly or indirectly to shedding of the E-cadherin ectodomain (Lochter et al., 1997). Here we directly test the activities of both stromelysin-1 and matrilysin with respect to
their ability to cleave E-cadherin. Matrilysin, which has a substrate specificity similar to stromelysin, is distinguished by its expression in well-differentiated epithelial cells, with this expression being linked to E-cadherin function (Borchers et al., 1997). Matrilysin is expressed in carcinomas of the gastrointestinal tract, prostate and breast and has been shown to be important for both tumor formation and tumor cell invasion (Rudolph-Owen et al., 1998; Wilson et al., 1996; Witty et al., 1994). Stromelysin, in contrast to matrilysin, is primarily expressed in the stromal fibroblasts at tumor sites. Its expression in tumor cells has been related to the loss of epithelial differentiation, though it has been suggested that stromelysin activity may be causative in this epithelial-to-mesenchymal transition rather than a result of it (Sternlicht et al., 1999). These observations prompted us to examine whether matrix metalloproteinases cleave E-cadherin and to investigate the paracrine biological effects of the released E-cadherin.

MATERIALS AND METHODS

Cell culture

MDCKs src Cl2 are Madin-Darby canine kidney cells transformed with a temperature-sensitive mutant of the src oncogene. At the non-permissive temperature for p60src activity (40°C), the cells have a functional E-cadherin/catenin complex. At the permissive temperature (35°C), the complex is no longer functional. These cells were tested at 40°C, the temperature at which they have a functional adherens junction complex. The MDCK cells were maintained in DMEM (Life Technologies, Ghent, Belgium), supplemented with 10% FBS (Life Technologies) and 0.05% L-glutamine (Behrens et al., 1993). The MCF-7/AZ cell line is a variant of the human breast cancer cell family MCF-7, for which the function of E-cadherin has been extensively tested (Bracke et al., 1993). MCF-7 puro and MCF-7 TIMP-2 Cl2 are Madin-Darby canine kidney cells transformed with a temperature-sensitive src plasmid. In this plasmid, the 5′ promoter is driving the expression of the puromycin resistence gene and a temperature-sensitive mutant of the src oncogene. At the non-permissive temperature for p60src activity (40°C), the cells have a functional E-cadherin/catenin complex. At the non-permissive temperature (35°C), the complex is no longer functional. These cells were tested at 40°C, the temperature at which they have a functional adherens junction complex. The MDCK cells were maintained in DMEM (Life Technologies, Ghent, Belgium), supplemented with 10% FBS (Life Technologies) and 0.05% L-glutamine (Behrens et al., 1993). The MCF-7/AZ cell line is a variant of the human breast cancer cell family MCF-7, for which the function of E-cadherin has been extensively tested (Bracke et al., 1993). MCF-7 puro and MCF-7 TIMP-2 Cl23 and Cl24 are MCF-7 cells infected with retrovirus containing a pBabe Puro control plasmid with the puromycin resistance gene only or a pBabe Puro TIMP-2 (tissue inhibitor of metalloproteinases-2) plasmid. In this plasmid, the 5′ Moloney murine leukemia virus LTR is driving the expression of TIMP-2 and an SV40 early promoter is driving the expression of the puromycin resistance gene (Noë et al., 1999). MCF-7 cells were cultured in a mixture (1/1) of Ham’s F12 and DMEM (Life Technologies) supplemented with 10% FBS and 0.05% L-glutamine. All media contained 100 i.u./ml penicillin and 0.1 mg/ml streptomycin.

Antibodies and chemicals

DECMA-1, a rat monoclonal antibody raised against the murine embryonal carcinoma cell line PCC7 Aza RL, recognizes the extracellular domain of murine and canine E-cadherin (Sigma, St Louis, MO, USA). HEC1D-1 is a murine monoclonal antibody recognizing the extracellular domain of human E-cadherin (Takara, San Diego, CA, USA). A rabbit polyclonal pan-cadherin antibody was raised using the synthetic peptide C-RFKKLADMYGGGEDD (provided by J. Vandeckerkchove, Department of Biochemistry, VIB, Ghent, Belgium) corresponding to a conserved sequence in the cytoplasmic tail of classical cadherins. The peptide was coupled to Keyhole limpet haemocyanin via the N-terminal cysteine and used for 4 cycles of intradermal and intramuscular immunization. The immune serum was affinity-purified by fast protein liquid chromatography (Pharmacia, Uppsala, Sweden) with the peptide bound to a p-hydroxymercuribenzoate matrix (Sigma). The immune serum recognizes bands corresponding with E-cadherin, N-cadherin and placental cadherin (P-cadherin) on western blot. We also used the mouse monoclonal anti-E-cadherin (Transduction Laboratories, Lexington, KY, USA), which recognizes the cytoplasmic tail of E-cadherin. Human recombinant matrilysin, stromelysin-1 and gelatinase B were obtained from Chemicon (Temecula, CA, USA) and trypsin from Sigma. For incubation with source cultures, matrilysin and stromelysin-1 were used at a concentration of 100 ng/ml diluted in serum-free medium. To test the effect on purified E-cadherin, 10 or 100 ng matrilysin, stromelysin or gelatinase B were dissolved in 50 μl PBS. Phorbol-12-myristate-13-acetate (PMA, Sigma) was used at concentrations of 10 and 100 ng/ml medium. Stock solutions of PMA were made at 1 mg/ml in DMSO.

Metabolic labeling

Pulse-chase experiments were performed as described (Shore and Nelson, 1991). Briefly, MDCKs src Cl2 cells were incubated for 30 minutes in methionine-free DMEM (Life Technologies) and pulse labeled for 10 minutes with 125 μCi/ml [35S]methionine (ICN, Costa Mesa, CA, USA). To chase labeled proteins, cells were incubated in DMEM containing a 5,000-fold excess of unlabeled methionine for the times indicated. The medium was removed and the cells were lysed with PBS containing 1% NP-40, 1% Triton-X 100 and the following protease inhibitors (all from Sigma): phenylmethyl-sulfonyl fluoride (1.72 mM), leupeptin (21 mM), aprotonin (10 μg/ml). Immunoprecipitations were performed with both conditioned medium and cell lysate.

Immunoprecipitation and western blotting

Equal amounts of protein or conditioned medium from equal cell numbers were incubated with an antibody for 3 hours at 4°C followed by protein G-Sepharose 4 fast-flow beads (Pharmacia) for 1 hour. Precipitated proteins were dissolved in sample buffer (Laemmli, 1970), boiled in the presence of 5% 2-mercaptoethanol and separated by SDS-PAGE. Proteins were transferred onto Immobilon-P membrane (Millipore Corp., Bedford, MA, USA). Blots containing [35S]methionine-labeled proteins were exposed to an autoradiographic film. For immunostaining, blots were quenched with 5% non-fat dry milk in PBS containing 0.5% Tween-20. The membranes were incubated with primary antibody, followed by three 5 minute washes with PBS, incubation with horseradish peroxidase-conjugated secondary antibodies. Proteins were detected using ECL reagent (Amersham Life Science, Buckinghamshire, UK) as substrate. Quantitation of western blots from at least three separate experiments was done by Quanti One (4.0; Bio-Rad, Ghent, Belgium).

Conditioned medium

Conditioned medium from MDCKs src Cl2 source cultures were obtained as follows. Cells were grown to confluence in tissue culture plastic vessels (Becton Dickinson, Franklin Lakes, NJ, USA), washed three times with serum-free DMEM (Life Technologies) and incubated for the indicated times at 40°C with serum-free DMEM to which matrilysin or stromelysin-1 were added. The media were harvested, centrifuged at 2,000 g for 15 minutes and passed through a 0.22 μm filter. 5E-CAD was removed from the conditioned medium as described (Noë et al., 1999). The conditioned medium was incubated for 3 hours with 2 μg DECMA-1 at 4°C, followed by protein G-Sepharose 4 fast-flow beads (Pharmacia) for 1 hour. The supernatant was incubated a second time for 3 hours with 2 μg DECMA-1 and 1 hour with protein G-Sepharose 4 fast-flow beads and finally passed through a 0.22 μm filter. Conditioned medium was diluted 1:2 in serum containing DMEM or in aggregation buffer before use.

Proteolytic cleavage of E-cadherin

The presence of sE-CAD in conditioned medium was examined by SDS-PAGE of the complete medium or of a DECMA-1 immunoprecipitate, followed by immunoblotting. Direct cleavage of E-cadherin was examined by treatment of pan-cadherin immunoprecipitates on beads. After three washes with PBS, the beads
Proteolysis of E-cadherin by MMPs

were incubated for 1 hour at 37°C in 50 μl PBS containing 10 ng or 100 ng matrilysin or stromelysin, or 100 ng gelatinase B. Supernatant and beads were separately dissolved in sample buffer, boiled in the presence of 5% 2-mercaptoethanol and separated by SDS-PAGE. Proteins were transferred onto Immobilon-P membrane (Millipore Corp.) and immunostained.

Collagen invasion assay

Type I collagen (Upstate Biotechnology, Lake Placid, NY, USA) was dissolved in bicarbonate buffer-containing DMEM with 1 M NaOH to neutralize the solution. 1.2 ml samples were poured into a 6-well plate and incubated overnight at 37°C for gelation. Cells, taken from cultures that had not been used for the preparation of conditioned medium, were seeded on top of the collagen gel either untreated, or in the presence of matrilysin or stromelysin-1 or conditioned medium of cells that had been treated with matrilysin, stromelysin or not. After 24 hours of incubation, the number of cells that invaded into the gel was monitored under a microscope with a computer-controlled step motor (Vakaet et al., 1991). The invasion index was calculated as the number of cells inside the gel over the total number of cells.

Aggregation assay

Cell-cell adhesion was numerically evaluated in an aggregation assay as described previously (Bracke et al., 1993). Briefly, cells were detached by collagenase A treatment (Boehringer Mannheim) followed by trypsin, both in the presence of 0.04 mM Ca²⁺. E-cadherin expression on the cell surface was conserved as demonstrated by flow cytometry. The cells were allowed to aggregate on a Gyrotory shaker (New Brunswick Scientific, New Brunswick, NJ, USA) at 80 rounds per minute for 30 minutes in an aggregation buffer containing 1.25 mM Ca²⁺, 0.1 mg DNase/ml, 10 mM Hepes and 0.1% BSA and equilibrated at pH 7.4. Cell aggregation was measured with an LS particle size analyser (LS 200, Coulter Electronics, Luton, England) after 0 and 30 minutes of aggregation. The relative volume as a function of the particle size was used as an index of aggregation (Noë et al., 1999).

Statistics

All experiments were performed at least twice. Student’s t-test was used for statistical analysis of the collagen invasion assay, and Kolmogorov-Smirnov statistics to analyze the differences between the cumulative distribution curves obtained in the fast aggregation assay.

RESULTS

Turnover of E-cadherin

In order to observe the time course of E-cadherin ectodomain shedding, MDCKTs.srcC12 cells at 40°C were pulse labeled and chased for the times indicated in Fig. 1. Immunoprecipitation of E-cadherin shows that the protein is synthesized as a 135 kDa precursor molecule which is processed within 15 minutes to the mature 120 kDa molecule (Fig. 1A). The asterisk indicates the 135 kDa E-cadherin precursor; arrows indicate E-cadherin and the associated catenins, namely α-catenin, β-catenin and γ-catenin (plakoglobin) in the cell lysate and sE-CAD in the medium.

Since the phorbol ester PMA has been shown to upregulate proteolytic activity from a variety of cells (Arribas et al., 1996), we tested its ability to enhance the production of sE-cad. Treatment of MCF-7/AZ cultures with PMA enhanced the amount of sE-cad in the media in a concentration-dependent manner, indicating that the activation of protein kinase C

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[Fig. 1. E-cadherin synthesis and release of sE-CAD from the cell surface. MDCKTs.srcC12 cells at 40°C were metabolically labeled for 10 minutes with [³⁵S]methionine and then chased with an excess of unlabeled methionine. Medium was taken off and cultures were extracted at various times during the chase period (0, 0.25, 0.5, 1, 2, 4, 6, 8 hours). E-cadherin was immunoprecipitated from the cells (A) and the medium (B) with DECMA-1 and separated by SDS-PAGE followed by blotting and exposure for fluorography. The asterisk indicates the 135 kDa E-cadherin precursor; arrows indicate E-cadherin and the associated catenins, namely α-catenin, β-catenin and γ-catenin (plakoglobin) in the cell lysate and sE-CAD in the medium.]

[Fig. 2. Effect of PMA on the release of sE-CAD into the medium. MCF-7/AZ source cultures were incubated in serum-free medium for 24 hours either untreated (lane 1) or in the presence of 10 ng/ml (lane 2) or 100 ng/ml (lane 3) PMA. After 24 hours, the medium was harvested and E-cadherin fragments were immunoprecipitated with HECD-1, separated by SDS-PAGE, blotted and immunostained with HECD-1. Numerical values represent percentages of untreated cultures as measured by Quanti One.]
Matrilysin and stromelysin-1 release sE-CAD from the cell surface

MDCKts.srcCl2 source cultures were treated for 2 hours with matrilysin, stromelysin-1 or trypsin, which, in the presence of Ca\(^{2+}\), cleaves E-cadherin to release an 80 kDa fragment into the medium (Takeichi, 1991). Both matrilysin and stromelysin-1 treatments stimulated the release of an E-cadherin fragment that was of equivalent size to the fragment constitutively released at low levels by the untreated cells (Fig. 4). Trypsin released an E-cadherin fragment slightly smaller than sE-CAD (Fig. 4).

sE-CAD production by matrilysin or stromelysin-1 treatment could be due to direct cleavage of E-cadherin by the proteases or to the activation of a common enzymatic cascade. To distinguish between these possibilities, we purified both human and canine E-cadherin from MCF-7/AZ (Fig. 5A,B) and MDCKts.srcCl2 (Fig. 5C,D) cells by immunoprecipitation with an antibody against the cytoplasmic domain of cadherins. E-cadherin immobilized on protein G-sepharose beads was treated with different concentrations of active forms of matrilysin, stromelysin-1, gelatinase B or with trypsin in the presence of Ca\(^{2+}\). Cleavage of E-cadherin was apparent by decreasing amounts of full-length E-cadherin retained on the beads and by the appearance of lower molecular mass bands both in the supernatant and on the beads. Matrilysin and stromelysin-1 treatments lead to the appearance in the supernatants of bands of approximately 80 kDa (Fig. 5A). Matrilysin releases two sE-CAD fragments with a slightly higher molecular mass than the stromelysin-cleaved sE-CAD. Beads treated with matrilysin or stromelysin retained bands of 40 kDa and 45 kDa, respectively, consistent with the size of the bands released into the supernatant (Fig. 5B).

In the supernatant of canine E-cadherin loaded beads, the two E-cadherin fragments released by matrilysin or stromelysin-1 have an identical molecular mass, higher than the corresponding human E-cadherin fragments (Fig. 5C) or than the canine sE-CAD released by trypsin treatment. As was seen for human E-cadherin, the amount of full-length E-cadherin decreased with increasing amounts of protease. A 40 kDa fragment appeared in the bead fraction following matrilysin treatment, similar to what was observed with human E-cadherin. However, the intracellular domain fragment was not detectable with stromelysin-1 treatments, suggesting the possibility of undetectable smaller fragments resulting from the presence of cryptic stromelysin-1 cleavage sites in this domain.

SE-CAD released by matrilysin and stromelysin-1 induces invasion and inhibits aggregation

To test whether sE-CAD released by matrilysin or stromelysin-1 could influence the invasion-suppressor function of E-cadherin, we examined the effect of conditioned medium derived from matrilysin or stromelysin-1 treated MDCKts.srcCl2 source cultures in a collagen type I invasion assay. Conditioned media of MDCKts.srcCl2 treated with matrilysin or stromelysin-1 for 2 hours were capable of strongly inducing the invasion of MDCKts.srcCl2 cells, whereas conditioned medium of untreated source cultures had no effect (Fig. 6A). As a positive control, cells were treated with 48-hour conditioned medium from the MDCKts.srcCl2 source cultures, shown previously to stimulate invasion in an sE-CAD dependent fashion. Our previous results indicate that induction of invasion occurs in an sE-CAD concentration-dependent manner (Noé et al., 1999). Invasion indices observed with conditioned media from 2 hour matrilysin or

![Graph](image-url)
Proteolysis of E-cadherin by MMPs

stromelysin-1 treated MDCKts.srcC12 source cultures were similar to those seen with the 48 hour conditioned medium containing constitutively shed sE-CAD, illustrating the strong effect of protease activity. To ensure that the increased invasion was due to sE-CAD and not a direct result of the added proteases acting on the collagen gel, sE-CAD was selectively removed. Immunodepletion of the sE-CAD from the conditioned media completely abrogated the invasion-inducing activity of the media (Fig. 6B). Moreover, addition of matrilysin or stromelysin-1 to unconditioned medium did not induce invasion (data not shown). Since the invasion assay was performed with serum-containing medium, we presume that MMP activity was neutralized due to the presence of the general proteinase inhibitor alpha 2-macroglobulin (Woessner, 1991).

Because the E-cadherin/catenin complex is also responsible for aggregation of epithelial cells, we tested in an aggregation assay the effect of the sE-CAD released by matrilysin or stromelysin-1 (Fig. 7). Aggregation was inhibited by the conditioned medium from source cultures treated with matrilysin (Fig. 7A) or stromelysin-1 (Fig. 7B) for 2 hours and not by conditioned medium of source cultures to which no proteinases were added (data not shown). When E-cadherin fragments were removed from the medium by immunoprecipitation, there is no longer inhibition of aggregation (Fig. 7). Together, these data indicate that the E-cadherin fragments released by direct cleavage by either matrilysin or stromelysin-1 are capable of inhibiting aggregation and inducing invasion of epithelial cells in a paracrine manner.

**Fig. 5.** Direct cleavage of E-cadherin by matrilysin and stromelysin-1. E-cadherin was immunoprecipitated from MCF-7/AZ cells (A,B) with a polyclonal pan-cadherin antibody. The beads were incubated at 37°C in 50 µl PBS only (lane 1), containing 10 ng matrilysin (lane 2), 100 ng matrilysin (lane 3), 10 ng stromelysin-1 (lane 4), 100 ng stromelysin-1 (lane 5), 100 ng gelatinase B (lane 6) or with 0.05% trypsin in the presence of 0.04 mM Ca²⁺ (lane 7). Beads and supernatant were separated, proteins were resolved by SDS-PAGE, blotted and immunostained with HECD-1 for the supernatant (A) and anti-E-cadherin, recognizing the cytoplasmic tail, for the beads (B). (C,D) E-cadherin was also immunoprecipitated from MDCKts.srcC12 cells with a pan-cadherin antibody and the beads were either untreated (lanes 1 and 6) or treated with 10 ng matrilysin (lane 2), 100 ng matrilysin (lane 3), 100 ng gelatinase B (lanes 4 and 9), 0.05% trypsin in the presence of 0.04 mM Ca²⁺ (lane 5), 10 ng stromelysin-1 (lane 7) or 100 ng stromelysin-1 (lane 8). Proteins in the supernatant or on the beads were separated by SDS-PAGE, blotted and immunostained with DECMA-1 (C) or anti-E-cadherin (D), respectively. •, indicates sE-CAD released in the supernatant; *, indicates full-length E-cadherin retained on the beads; †, indicates the fragment, corresponding to the cytoplasmic tail of E-cadherin, retained on the beads.
DISCUSSION

E-cadherin is a cell-cell adhesion molecule having invasion suppressor activities. Disturbance of the E-cadherin/catenin complex during tumor progression is correlated with the onset of an invasive phenotype (Bracke et al., 1996) although other cadherins may be crucial as well (Nieman et al., 1999b). Multiple mechanisms of inactivation of the E-cadherin/catenin complex are exploited by tumors including genetic mutation, gene inactivation through methylation, and alteration of the associated molecules, the catenins. Here, we provide evidence that inhibition of E-cadherin function also can be mediated through the shedding of its ectodomain as a result of cleavage by the metalloproteinases, matrilysin and stromelysin-1.

In contrast to the loss of E-cadherin function, it is the gain of metalloproteinase activity that is frequently associated with the onset of tumor cell invasion. The ability of MMPs to enhance tumor cell invasion has been thought to be a result of the degradation of the extracellular matrix, a natural barrier to invasion and metastasis. However, recent evidence indicates that both membrane-associated and soluble MMPs can mediate cell growth, apoptosis and signal transduction (Chambers and Matrisian, 1997; Noël et al., 1997). These unexpected properties of MMPs have been linked to the MMP cleavage of the ectodomain of transmembrane proteins such as growth factor ligands and receptors. In this study, we show that MMP-mediated induction of cellular invasion also can be independent of direct matrix degradation and, instead, a result of the release of E-cadherin fragments. This cleavage event can be mediated by either matrilysin or stromelysin, but shows some degree of enzyme specificity in that purified gelatinase B did not recognize E-cadherin as a substrate.

The fact that both matrilysin and stromelysin-1 can cleave E-cadherin is not completely unexpected as these enzymes share similar substrate specificity. Studies with synthetic peptides have shown that matrilysin preferentially cleaves proteins with a leucine at the P1 and P2 positions of the cleavage site (Smith et al., 1995). The same motif was also

Fig. 6. Induction of invasion of MDCKts.srcCl2 cells into collagen type I by conditioned medium containing sE-CAD. (A) The MDCKts.srcCl2 cells were seeded on top of collagen type 1 gel and incubated at 40°C for 24 hours in the presence of conditioned medium that was obtained by incubating an MDCKts.srcCl2 source culture for 0 (I) or 2 (II) hours either in the absence (open bars) or in the presence of 100 ng/ml matrilysin (gray bars) or 100 ng/ml stromelysin (black bars); conditioned medium harvested from untreated source cultures after 48 hours and containing spontaneously released sE-CAD (III) served as a positive control. (B) Invasion of MDCKts.srcCl2 cells at 40°C treated with conditioned medium of untreated source cultures (I) or conditioned medium from matrilysin (II) or stromelysin-1 (III) treated source cultures; hatched bars indicate removal of sE-CAD from conditioned medium by immunoprecipitation with DECMA-1. The invasion indices were calculated as the number of cells inside the gel over the total number of cells.

Fig. 7. Inhibition of aggregation of MDCKts.srcCl2 cells by sE-CAD released by matrilysin or stromelysin-1. MDCKts.srcCl2 cells at 40°C were detached under E-cadherin-saving conditions, suspended in aggregation buffer and allowed to aggregate for 30 minutes. Cell aggregation was measured by particle size counting either after 0 or 30 minutes of aggregation. Aggregation of MDCKts.srcCl2 cells at 40°C was inhibited by sE-CAD containing conditioned medium of source cultures treated (open triangles) with matrilysin (A) or stromelysin-1 (B). When sE-CAD was removed from the conditioned medium by immunoadsorption, aggregation was no longer inhibited (solid line). Peak positions of untreated cells were 19 μm after 0 minutes and 102 μm after 30 minutes of aggregation.
shown to be a relatively good substrate for stromelysin-1 (Smith et al., 1995). The extracellular portions of both human and canine E-cadherin contain several possible cleavage sites close to the plasma membrane, which would correspond to the sizes of the released fragments. The presence of more than one putative cleavage site could explain the two bands that are released into the supernatant following treatment with matrilysin or stromelysin-1 of E-cadherin isolated on beads or on the cell surface (see Figs 4 and 5).

The enzyme responsible for constitutive shedding of sE-CAD in MCF-7 cells is unknown, although of the MMPs examined in these experiments matrilysin is the most obvious candidate. Matrilysin, but not stromelysin-1, gelatinase A or gelatinase B, can be detected in concentrated conditioned medium from MCF-7 cultures (our unpublished results). However, with TPA treatment, stromelysin-1 protein is induced (our unpublished results). Thus, matrilysin could be responsible for the TIMP-2 inhibitable constitutive release of sE-CAD from MCF-7 cultures, whereas the combination of matrilysin and stromelysin-1 could be responsible for E-cadherin cleavage upon TPA stimulation. However, the expression of these proteinases cannot fully explain the presence of sE-CAD that is detected at 22 hours in TIMP-2 expressing cultures, suggesting that there are other proteinases that are not inhibited by TIMP-2 and that can cleave E-cadherin. Trypsin, a serine protease, can release a similar E-cadherin fragment from the cell surface (Fig. 5C), suggesting that other classes of proteinases can be involved. Also, the ADAM family of cell surface metalloproteinases with disintegrin domains, which are not inhibitable by TIMP-2, have been shown to be ectodomain sheddases for a variety of cell surface molecules, including tumor necrosis factor α, L-selectin, and transforming growth factor α (Peschon et al., 1998).

Interestingly, we have shown that it is not the cleavage event itself that is responsible for the resulting inhibition of aggregation and increased invasive properties of the cell, but instead it is an activity of the soluble ectodomain released from the adherens junction complex. That is, when the shed ectodomain is immunodepleted from the matrilysin or stromelysin-1 treated conditioned media, the media lose their capability of inhibiting aggregation and inducing invasion in untreated cells. Disruption of cell-cell contact caused by sE-CAD is presumably due to its ability to mimic an intact E-cadherin molecule by interacting with E-cadherin anchored to the cell surface. However, this simple physical disruption of cell-cell contact is insufficient to explain the ability of sE-CAD to induce invasion of cancer cells. More likely is the stimulation of a signal transduction pathway, either directly by sE-CAD interactions with E-cadherin or indirectly by the disruption of cell/cell contact.

Although our data reveal the invasion-promoting effect of sE-CAD, it is possible that the remnant 40 kDa transmembrane fragment may have a similar function. There is evidence in the literature for a function with such a fragment of receptor tyrosine kinases, where removal of the ectodomain results in constitutive activation of the kinase activity present in the remaining fragment (Cabrera et al., 1996; Rodrigues and Park, 1994). It has been reported that trypsin-mediated removal of the E-cadherin ectodomain induces tyrosine phosphorylation of β-catenin (Takahashi et al., 1997), a protein that associates with the E-cadherin cytoplasmic tail forming part of the adherens complex. Mutant cadherins containing the cytoplasmic tail, thus mimicking the 40 kDa remnant fragment, exert dominant-negative effects when expressed in Xenopus embryos (Kintner, 1992), in cultured epithelial cells (Fujimori and Takeichi, 1993; Nieman et al., 1999a), and in mouse intestinal epithelia (Hermiston and Gordon, 1995a; Hermiston and Gordon, 1995b). Such fragments have also been shown to directly block β-catenin signalling (Crawford et al., 1999; Orsulic et al., 1999). E-cadherin cleavage may therefore promote invasion through both an sE-CAD fragment acting in a paracrine manner and a 40 kDa remnant fragment that can act in an autocrine manner.

Since sE-CAD is also found in human cancers, our present in vitro experiments may also be relevant for invasion in vivo (Katayama et al., 1994; Banks et al., 1995; Griffiths et al., 1996; Gofuku et al., 1998). In such cancers, sE-CAD might be generated by the enhanced proteolysis associated with invasion in many experimental and clinical cancers (Chambers and Matrisian, 1997).

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