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Inhibition of adhesion and induction of epithelial cell invasion by HAV-containing E-cadherin-specific peptides

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SUMMARY

The E-cadherin/catenin complex, an organizer of epithelial structure and function, is disturbed in invasive cancer. The HAV (histidine alanine valine) sequence in the first extracellular domain of E-cadherin is crucial for homophilic interactions between cadherins. We report that specific peptides containing an HAV sequence interfere with the functions of the E-cadherin/catenin complex. Cells either expressing specific cadherins or not were challenged with both cadherin and noncadherin peptides comprising a central HAV sequence. Specific E-cadherin peptides inhibited cell aggregation, disturbed the epithelial morphotype and were able to stimulate invasion of cells expressing E-cadherins. Conditioned medium, containing E-cadherin fragments, also stimulated invasion in contrast to conditioned medium from which the E-cadherin fragments were removed. Our studies show that E-cadherin functions are inhibited by homologous proteolytic HAV-containing fragments that are released in an autocrine manner and subsequently inhibit the E-cadherin/catenin complex. In this way such cadherin fragments may induce and support cancer invasion.

Key words: E-cadherin/catenin, HAV peptide, Cell-cell adhesion, Invasion, Cadherin fragment

INTRODUCTION

Cadherins are a family of closely related calcium-dependent cell-cell adhesion and signal-transducing integral membrane glycoproteins (Takeichi, 1995). They form complexes with the catenins, intracellular proteins of the armadillo family. The E-cadherin/catenin complex is necessary for development and maintenance of epithelial organisation. Dysfunction of the E-cadherin/catenin complex has been correlated with malignancy as evidenced by tumor progression, loss of differentiation, invasion, metastasis and poor prognosis (Behrens et al., 1989; Vleminckx et al., 1991; Takeichi et al., 1993; Birchmeier et al., 1993; Mareel et al., 1994). Cadherins are expressed in a tissue-specific manner as exemplified by the classical cadherins: E-(epithelial), P-(placental) and N-(neural) cadherin (Takeichi, 1991). The extracellular part of E-cadherin contains a tandemly repeated sequence and consists of 5 domains forming an elongated monomeric structure in the presence of calcium (Pokutta et al., 1994). The first amino (NH_2)-terminal extracellular repeat of the classical cadherins contains a highly conserved HAV (histidine alanine valine) sequence, that is crucial for homophilic cadherin interactions. The exact mechanism of this interaction is, however, still a matter of debate. Indeed, the structure of the first extracellular repeat of E-cadherin as determined by nuclear magnetic resonance, did not reveal dimerisation of E-cadherin fragments (Overduin et al., 1995). This is in contrast to crystallographic analysis revealing that the NH_2-terminal domain of N-cadherin has a parallel dimer interface, which links two cadherin molecules on the same cell through hydrophobic interactions. This cadherin dimer binds to another cadherin dimer on a neighbouring cell, so forming an adhesion zipper (Shapiro et al., 1995). Crystallographic analysis of the first two repeats of E-cadherin also revealed dimers, linked together by calcium ions (Nagar et al., 1996), and this structure was confirmed biochemically by Koch et al. (1997). For homophilic binding of *Xenopus* C-cadherin, lateral dimerisation is also required (Brieher et al., 1996).

The high conservation of the HAV sequence in cadherins suggests a specific function for this amino acid motif. Next to classical cadherins, it occurs also in many other proteins some of which are possibly implicated in cellular interactions, namely as: FGFR (fibroblast growth factor receptor) (Williams et al., 1994), influenza hemagglutinins (Byers et al., 1992) and ECSODB (rat extracellular superoxide dismutase B) (Willems et al., 1993).

E-cadherin functions can be downregulated at multiple levels, for instance: by genomic mutations (Becker et al., 1993; Berx et al., 1996), by tyrosine phosphorylation of β-catenin (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et

al., 1993) and by disturbance of intracellular associations with catenins (Hinck et al., 1994; Näthke et al., 1994). In addition to those ways of functional regulation, protein fragments or peptides that comprise the HAV sequence may also display regulatory activity when applied extracellularly. Indeed, a soluble 80 kDa fragment, purified from the medium of MCF-7 cell cultures, induced scattering of murine mammary tumor epithelial cells (Wheelock et al., 1987). This effect was reversible and could be inhibited by an antibody against gp120, identical to E-cadherin, from human gestational choriocarinoma JAR cells. In contrast, neither a glycoprotein (gp84) trypsinized from murine embryonal carinoma PCC4 cells (Hyafil et al., 1980) nor the recombinant 80 kDa fragment, harvested from a baculovirus expression system (both derived from E-cadherin), did have decompacting activity (Herrenknecht and Kemler, 1993). An N-cadherin-specific HAV-containing decapeptide inhibited compaction of eight-cell-stage mouse embryos and outgrowth of rat neurites on astrocytes, two processes that are mediated by cadherins (Blaschuk et al., 1990). Neurite outgrowth was also inhibited by a 12-mer HAV-containing peptide with the sequence found in FGFR1 or FGFR2 indicating that N-cadherin may signal also through FGFR (Williams et al., 1994). N-cadherin dependent myoblast fusion was inhibited by an N-cadherin peptide (Mège et al., 1992). We found previously that N-cadherin- and ECSODB-specific HAV-containing decapeptides inhibit N-cadherin mediated aggregation, whereas E-cadherin- and P-cadherin-specific HAV peptides block the aggregation of cells expressing, respectively, E-cadherin and P-cadherin (Willems et al., 1995). E-cadherin-specific 17-mer HAV peptides inhibited E-cadherin mediated fusion of monocytes during the formation of multinucleated bone-resorbing osteoclasts (Mbalaviele et al., 1995).

The inhibition of cadherin functions by the above mentioned peptides suggests that cadherin fragments containing an HAV sequence may stimulate invasion, a process that is counteracted by the expression of a functional E-cadherin/catenin complex (Vleminckx et al., 1991). We, therefore, further investigated the influence of synthetic 10-mer and 20-mer HAV peptides on cadherin-related functions of different cell lines. We were able to demonstrate that synthetic HAV peptides inhibited E-cadherin mediated cellular interactions and induced invasion in different in vitro systems. Conditioned medium of E-cadherin expressing cells, containing E-cadherin fragments, was also able to induce invasion while conditioned medium from which the E-cadherin fragments were removed by immunoprecipation did no longer induce invasion. These results suggest that extracellular cadherin fragments induce loss of cell-cell adhesion and lead to tumor cell invasion.

MATERIALS AND METHODS

Cell culture

Cells expressing functional and species specific E-cadherin were used to test the influence of the HAV peptides on E-cadherin. MDCKts.*src*Cl2 (Behrens et al., 1993) are Madin-Darby canine kidney (MDCK) cells transformed with a temperature sensitive *src* oncogene. At the non-permissive temperature for $p60^{v\text{-}src}$ activity (40.5°C), the cells have a functional E-cadherin/catenin complex. At the *src*-permissive temperature (35°C), the E-cadherin/catenin complex loses its function. MDCK-*ras*-e c5 cells were grown from microcapsules injected intraperitoneally in nude mice; they express functional canine E-cadherin (Vandenbossche et al., 1994). NM-f-*ras*-TD1 is a cell line derived from an invasive tumor, formed after subcutaneous injection into nu/nu mice of a fibroblast-like subclone from a murine mammary gland cell line (NMuMG) transfected with the *ras* oncogene (Vleminckx et al., 1991). NM-f-*ras*-TD1 cells do not express E-cadherin and were used as a negative control in the present experiments. The NM-f-*ras*-TD-CAM5 cell line was derived from NM-f-*ras*-TD1 cells after successful cotransfection with the plasmid pBATEM2, containing the murine E-cadherin cDNA, and with a plasmid conferring resistance to mycophenolic acid. NM-e-*ras* is an epithelioid NMuMG cell clone transfected with the *ras* oncogene but still expressing murine E-cadherin. NM-f-*ras*-TD-CAM5 and NM-e-*ras* cells both have a functional E-cadherin/catenin complex (Vleminckx et al., 1991). The MCF-7/AZ cell line is a variant of the human breast cancer cell family MCF-7, that has a functional E-cadherin/catenin complex (Bracke et al., 1993). 2B2 and ARM cells are S180 mouse sarcoma cells transfected with and expressing, respectively, chicken E-cadherin and chicken N-cadherin (Mège et al., 1988). VE-CHO Cl5a cells are CHO cells transfected with and expressing VE-cadherin (Breviario et al., 1995).

All cells of the MDCK cell family and VE-CHO Cl5a were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Ghent, Belgium), supplemented with 10% (v/v) fetal bovine serum (FCS, Life Technologies) and 0.05% (w/v) L-glutamine. For cells of the NMuMG family, DMEM was supplemented with 10% fetal bovine serum and 10 μg/ml insulin (Sigma Chemical Company, St Louis, MO). MCF-7/AZ cells were cultured in EMEM (Earl's modified Eagle's medium, Life Technologies) supplemented with 5% fetal bovine serum and 6 ng/ml bovine insulin. S180, 2B2 and ARM cells were maintained in DMEM supplemented with 15% FCS and 0.05% L-glutamine. All media contained 100 units/ml penicillin and 0.1 mg/ml streptomycin.

Peptides, conditioned medium, antibodies and reagents

Different 10-mer and 20-mer HAV-comprizing peptides, similar to sequences in the amino-terminal part of different cadherins, were used in the present experiments as well as peptides with the sequence of other HAV-containing proteins. As controls a scrambled human E-cadherin 10-mer peptide (hu $E\text{-}CAD^{10scr}$) and 2 peptides containing the canine E-cadherin HAV flanking amino acids (c $E\text{-}CAD^{11flHAV}$ and c $E\text{-}CAD^{HAVfl10}$) were included in the experiments (Table 1).

The human E-cadherin (NH_2-LFSHAVSSNG-amide; hu $E\text{-}CAD^{10}$) differs in the HAV flanking sequences from dog and murine E-cadherin (LYSHAVSSNG) by only 1 amino acid and from chicken E-cadherin (LLSHAVSASG) by 3 amino acids. In the rat ECSODB (NH_2-REMHAVSRQV-amide; Ra SOD^{10}) a valine residue was substituted for an alanine (NH_2-REMHAASRQV-amide; Ra SOD^{10mut}). Conversely, in the human ECSODC, that contains an HAA instead of a HAV sequence (NH_2-GTLHAASQVQ-amide; hu SOD^{10}), an alanine was replaced by a valine (NH_2 GTLHAVSQVQ-amide; hu SOD^{10mut}). All 10-mer peptides (Innogenetics, Zwijnaarde, Belgium and Ansynth Service, Roosendaal, The Netherlands) were separated by HPLC to at least 95% purity. All 10-mer and 20-mer peptides were used at a concentration of 200 μg/ml. In our previous experiments (Willems et al., 1995), decapeptides exhibited a maximal effect at this concentration.

MB2 is a mouse monoclonal antibody raised against MCF-7/AZ cells, recognizing E-cadherin and with neutralizing effects (Bracke et al., 1993). DECMA-1 is a rat monoclonal antibody raised against the murine embryonal cell line PCC7 Aza RI and recognizing murine and canine E-cadherin (Sigma). Rabbit IgG recognizing chicken E-cadherin was kindly provided by R. M. Mège (Mège et al., 1988).

Conditioned medium of the MDCKts.*src*Cl2 cells was obtained

Table 1. Sequences of cadherin-derived or homologous peptides

Type	Species	Sequence	Acronym
E-cadherin	Human	LFS **HAV** SSNG	hu E-CAD[10]
	Murine	LYS **HAV** SSNG	
	Chicken	LLS **HAV** SASG	
	Canine	CAKYILYS **HAV** SSNGNAVED	c E-CAD[20]
		QLAKYILYS **HA**	c E-CAD[11flHAV]
		V SSNGNAVED	c E-CAD[HAVfl10]
		LHSNSVGFSA	hu E-CAD[10scr]
N-cadherin	Human	LRA **HAV** DING	hu N-CAD[10]
	Human	CARFHLRA **HAV** DINGNQVENG	hu N-CAD[20]
P-cadherin	Human	LFG **HAV** SENG	hu P-CAD[10]
ECSODB	Rat	REM **HAV** SRQV	Ra SOD[10]
ECSODC	Rat mutated	REM **HAA** SRQV	Ra SOD[10mut]
	Human	GTL **HAA** SQVQ	hu SOD[10]
	Human mutated	GTL **HAV** SQVQ	hu SOD[10mut]
FGFR1	Human	KKL **HAV** PAAK	FGFR1[10]
Hemagglutinin	Influenza virus strain A	LGH **HAV** SNGT	HA(HMIVF)[10]

EC SOD, extracellular superoxide dismutase; FGFR, fibroblast growth factor receptor; HA(HMIVF), haemagglutinin of influenza virus A strain; flHAV, amino acids flanking the HAV sequence at the left; HAVfl, amino acids flanking the HAV sequence at the right; scr, scrambled.

as follows. Cells were grown to confluence in 75 cm^2 tissue culture plastic vessels (Becton Dickinson, Franklin Lakes, NJ). The cells were washed 3 times with serum-free DMEM (Life Technologies) and incubated at 40°C with serum-free DMEM. After 48 hours of culture, the medium was harvested, centrifuged at 2,000 ***g*** for 30 minutes and filtered through a 0.22 μm filter to eliminate possible cell particles. To remove E-cadherin fragments from the conditioned medium, immunoprecipitations with DECMA-1 were performed. The conditioned medium was incubated for 3 hours with DECMA-1 at 4°C, followed by incubation with Protein G-Sepharose 4 fast flow beads (Pharmacia, Uppsala, Sweden) for 1 hour. The supernatant was incubated for a second time for 3 hours with DECMA-1 and 1 hour with Protein G-Sepharose 4 fast flow beads. The medium was recuperated and filtered through a 0.22 μm filter. The effect of conditioned medium containing E-cadherin fragments or not was tested in the collagen invasion assay at a dilution of 1:2.

To test the presence of E-cadherin fragments in the conditioned medium, precipitated proteins were dissolved in sample buffer (Laemmli, 1970), boiled in the presence of 5% (v/v) 2-mercaptoethanol and separated by 7.5% SDS-PAGE. Proteins were transferred onto Immobilon-P membrane (Millipore Corp., Bedford, MA). After quenching with 5% (w/v) non-fat dry milk in PBS containing 0.5% Tween-20, the membranes were incubated with DECMA-1 as primary antibody, followed by 3× 5 minutes washes and incubation with alkaline phosphatase-conjugated secondary antibody. Proteins were stained using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as substrate.

Fast aggregation assay

Cell-cell adhesion was numerically evaluated in an aggregation assay as described earlier (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 Ca^{2+}. This procedure was proven by flow cytometry to conserve E-cadherin expression on the cell surface. The cells were allowed to aggregate on a Gyrotory shaker (New Brunswick Scientific, New Brunswick, NJ) at 80 rounds per minute for 30 minutes in an aggregation buffer containing 1.25 mM Ca^{2+}, 0.1 mg DNase/ml, 10 mM Hepes (4-(2-hydroxyethyl)piperazine ethane-sulfonic acid) and 0.1% (w/v) bovine serum albumine (BSA) and equilibrated at physiological pH and osmolarity. The aggregation index was expressed as $1\text{-}N_{30}/N_{0,}$ where N_0 indicates the initial number of particles and N_{30} the number of particles after 30 minutes of aggregation. The number of particles was measured by a Coulter counter ZM (Coulter Electronics, Luton, England). Cell aggregation was also measured with an LS particle size analyser (LS 200, Coulter Electronics). Here, relative volume in function of the particle size was used as an index of aggregation (Vermeulen et al., 1995).

Slow aggregation assay

2×10^4 cells in 200 μl medium with or without peptide or antibody were seeded on top of 50 μl of a 0.66% (w/v) agar solution, that was poured into a 96-well plate (Nunc, Roskilde, Denmark) at 50°C and allowed to gel for 30 minutes at 4°C (Bracke et al., 1993). MDCKts.*src*Cl2 cells were incubated for 2 hours at 37°C followed by 22 hours at 40°C. Aggregate formation was scored under an inverted microscope with a ×10 objective (Leitz, Wetzlar, Germany) after 24 hours of incubation. Viability of the cells was checked by Trypan Blue exclusion.

Collagen invasion assay

Collagen G (type I solution, Seromed, Biochrom KG, Berlin, Germany) was dissolved at 0.22% (w/v) in bicarbonate buffer-containing DMEM. 1.2 ml aliquots were poured into a 6-well plate and incubated overnight at 37°C for gelation. Cells were seeded on top of the collagen gel with or without peptides or antibody. After 24 hours of incubation at temperatures of 35°C, 37°C or 40°C, depending on the cell line, the number of cells that invaded the gel as well as the depth of the invasive cells 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. The morphotype of the cells on top of the gel was examined after 9 days of further culture.

Chick heart invasion assay

Invasion into embryonic chick heart fragments was tested as described earlier (Mareel et al., 1979). Briefly, monolayer fragments of cultured cells were confronted with 9-day-old precultured embryonic chick heart fragments on a semi-solid agar medium. The confronting pairs were incubated for 24 hours and transferred into an Erlenmeyer flask with 1.5 ml of liquid culture medium. Peptide or antibody, if present, was replaced by a fresh solution after 2 days. Confronting pairs were fixed after 4 days, embedded in paraffin and serially sectioned for staining with hematoxylin-eosin and immunohistochemically with antisera either recognizing the chick heart or the confronting cells. Invasion was scored on serial histological sections as described earlier (Bracke et al., 1994). Confronting cells possessing a functional E-cadherin/catenin

complex form an epithelioid layer around the heart tissue and do not invade (Vleminckx et al., 1991).

Morphotype on solid substrate

For seeding on solid substrate, cells were detached with trypsin/EDTA (Life Technologies) from a nearly confluent monolayer, suspended and seeded at 1×10^4 cells per ml in a 24-well plate. Cultures were grown for about 3 days and when epithelioid islands were formed, peptide or antibody was added. The cultures were scored for scattering after 4 hours.

Statistics

All experiments were performed at least twice. Student's *t*-test was used for statistical analysis of the fast aggregation assay and of the collagen invasion assay. For all subjective observations (slow aggregation, morphology and invasion into chick heart) 3 independent observers scored coded samples.

RESULTS

Aggregation

E-cadherin dependent aggregation was examined in both a fast (30 minutes) assay under Gyrotory shaking (Table 2 and Fig. 1) and a slow (24 to 48 hours) assay under static conditions (Fig. 2). The Ca^{2+}-dependent fast aggregation of all cells expressing E-cadherin was inhibited by hu E-CAD[10] peptide.

Table 2. HAV-containing peptides or cadherin antibody inhibit aggregation and stimulate collagen invasion of cells expressing species-specific E-cadherins

	Cell type (E-cadherin species) amino acid sequence					
	Fast aggregation					
Treatment	MDCKts.*src*Cl2 (canine) LYS**HAV**SSNG	MCF-7/AZ (human) LFS**HAV**SSNG	NM-e-*ras* (murine) LYS**HAV**SSNG	NM-f-*ras*-TD-CAM5 (murine) LYS**HAV**SSNG	2B2 (chicken) LLS**HAV**SASG	VE-CHO Cl5a (human) LTAVIVDKQT
None	100±14	100±29	100±14	100±11	100±17	100±9
hu N-CAD[10]	99±6	84±5	84±5	99±3	91±8	95±4
hu N-CAD[20]	99±1					88±11
hu E-CAD[10]	**31±4**	**47±2**	**15±2**	**45±2**	**82±8**	91±7
c E-CAD[20]	**18±3**	**47±1**				86±10
hu P-CAD[10]		68±10	82±4	94±8	91±4	
Ra SOD[10]		89±8	80±2	101±3	94±4	
Ra SOD[10mut]		72±15			81±6	
hu SOD[10]		78±2			84±6	
FGFR1[10]			58±15	36±6		
Antibody	**11±4**	**2±2**	**9±8**	**8±9**	**5±1**	
	Collagen Invasion					
	MDCKts.*src*Cl2 (canine) LYS**HAV**SSNG	MDCK-*ras*-e c5 (canine) LYS**HAV**SSNG	NM-e-*ras* (murine) LYS**HAV**SSNG	NM-f-*ras*-TD-CAM5 (murine) LYS**HAV**SSNG	NM-f-*ras*-TD1 (none)	
None	100±20	100±25	100±15	100±10	100±13	
hu N-CAD[10]	81±27	80±39	94±22	57±15	92±21	
hu N-CAD[20]	149±41					
hu E-CAD[10]	**350±57**	**688±117**	**247±21**	**257±27**	126±11	
hu E-CAD[10scr]	113±23					
c E-CAD[20]	**1089±155**					
c E-CAD[11flHAV]	68±5					
c E-CAD[HAVfl10]	86±14					
hu P-CAD[10]		138±38	143±17	110±19	121±17	
Ra SOD[10]			85±21	87±15	84±9	
Ra SOD[10mut]			127±31	127±23	96±12	
hu SOD[10]			169±41	107±16	102±11	
hu SOD[10mut]			**256±37**	169±14*	100±15	
FGFR1 [10]			203±34*	104±11	110±19	
HA(HMIVF)[10]			199±24*	165±18*	110±11	
Antibody	**339±62**	**497±85**	**505±38**	**254±25**	98±20	

Numerical values indicate percentages of untreated ± s.d.; each experiment was repeated at least twice; bold means significantly different from untreated with $P<0.005$ or $*P<0.01$ (Student's *t*-test). hu E-CAD, human E-cadherin; c E-CAD, canine E-cadherin; hu N-CAD, human N-cadherin; hu P-CAD, human P-cadherin; Ra SOD, rat extracellular superoxide dismutase B; Ra SOD[10mut], mutated Ra SOD; hu SOD, human extracellular superoxide dismutase C; hu SOD[10mut], mutated hu SOD; FGFR1, fibroblast growth factor receptor 1; HA(HMIVF), hemagglutinin from influenza virus strain A; flHAV, peptide containing the amino acids at the left of the HAV sequence; HAVfl, peptide containing the amino acids at the right of the HAV sequence; scr, scrambled; the superscripts: figures indicate number of amino acids; mut, mutated; antibody, MB2, a-ch E-cadherin and DECMA-1, respectively, for human, chicken and canine or murine cells. Experiments with MDCKts.*src*Cl2 cells were done at 40°C. Activation of *src* at 35°C reduced fast aggregation to 16±10% and increased invasion to 321±55% of untreated cells at 40°C.

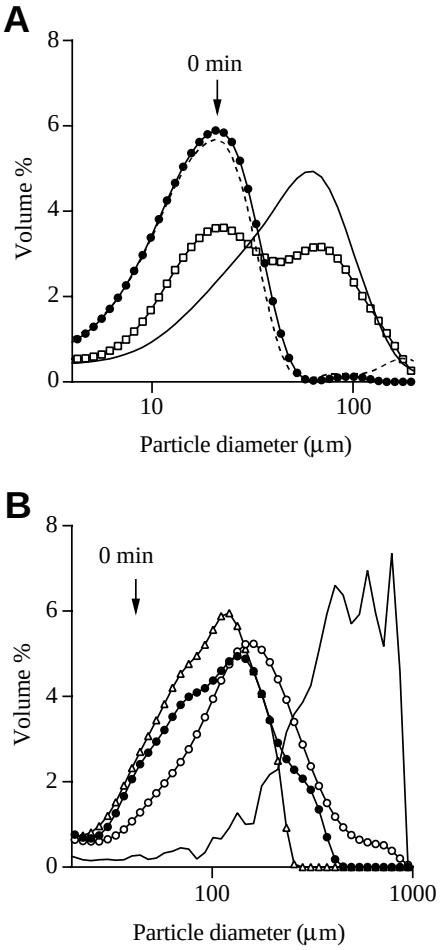

Fig. 1. Inhibition at 40°C of fast aggregation of MDCKts.*src*Cl2 cells by peptides hu E-CAD^{10} and hu N-CAD^{10} (A) and of MCF-7AZ cells by hu E-CAD^{10} and hu P-CAD^{10} (B). Cells were detached under E-cadherin-saving conditions, suspended in aggregation buffer and allowed to aggregate for 30 minutes. Cell aggregation was measured by particle size counting either after 0 minutes (peak positions are indicated with an arrow) or 30 minutes of aggregation (—). Aggregation of MDCKts.*src*Cl2 cells at 40°C (A) was inhibited by hu E-CAD^{10} (●) and DECMA-1 (--). Treatment with hu N-CAD^{10} (□) yields a biphasic curve, indicating inhibition of aggregation of a subset of MDCKts.*src*Cl2 cells. Aggregation of MCF7/AZ cells (B) was inhibited by hu E-CAD^{10} (●) and hu P-CAD^{10} (○) peptides. Combination of both peptides (△) showed additive inhibitory effects on MCF-7/AZ cell aggregation.

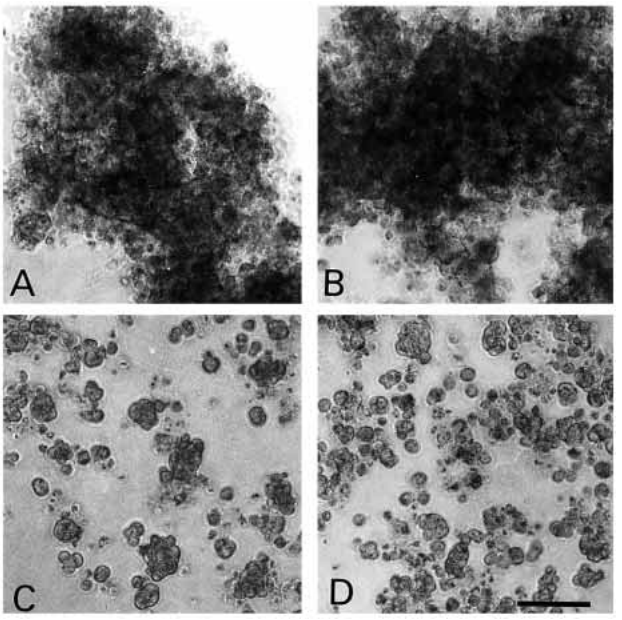

Fig. 2. Inhibition of aggregation of MDCKts.*src*Cl2 cells by antibody DECMA-1 and peptide hu E-CAD^{10} but not by hu N-CAD^{10}. Cells were seeded on a gelified agar medium and incubated at 37°C for 2 hours followed by incubation at 40°C for 22 hours without added reagents (A), treated with hu N-CAD^{10} peptide (B), hu E-CAD^{10} peptide (C) or treated with DECMA-1 antibody (D). Aggregate formation was scored after 24 hours of incubation. Addition of Trypan Blue at the end of the assay showed no dead cells. Bar, 100 μm.

Only the aggregation of 2B2 cells, expressing chicken E-cadherin with a larger difference in the HAV-flanking amino acids and aggregation of VE-CHO Cl5a, expressing VE-cadherin not having an HAV sequence, could not be inhibited by hu E-CAD^{10}. HAV decapeptides with other flanking sequences, e.g. hu N-CAD^{10} or hu P-CAD^{10}, did not significantly alter the aggregation index of cells expressing E-cadherin (Table 2). The FGFR1^{10} peptide (see Table 1) stands as an exception since it also inhibited aggregation of NMuMG cells expressing either the endogenous or an exogenous murine E-cadherin (Table 2) as well as ARM cells expressing chicken N-cadherin (data not shown). Also the 20-mer peptide c E-CAD^{20} inhibited aggregation of MDCKts.*src*Cl2 cells at 40°C.

Fast aggregation of MDCKts.*src*Cl2 cells was measured also with an LS particle size analyzer. We found inhibition of aggregation by 10-mer as well as 20-mer E-cadherin HAV peptides and by the DECMA-1 antibody (Fig. 1A). Treatment with N-cadherin peptide produced a biphasic curve, indicating inhibition of aggregation in a subset of MDCKts.*src*Cl2 cells, weakly expressing N-cadherin (data not shown). The minor effects of other cadherin peptides was also investigated in MCF-7/AZ cells. We also detected by western blotting, next to E-cadherin, expression of P-cadherin but not of N-cadherin (data not shown). By particle size counting, we found an inhibition of aggregation by hu P-CAD^{10} that was additive to hu E-CAD^{10} (Fig. 1B), while hu N-CAD^{10} had no significant effect (data not shown).

Inhibition by hu E-CAD^{10} peptide of E-cadherin-specific aggregate formation by MDCKts.*src*Cl2 cells at 40°C was also shown in the slow aggregation assay (Fig. 2).

Invasion into collagen type I

Induction of invasion by peptides and antibodies into collagen type I is shown in Table 2. At 40°C, invasion was induced in MDCKts.*src*Cl2, up to the level of invasion at 35°C, by the antibody DECMA-1, by hu E-CAD^{10} or c E-CAD^{20} but not by hu N-CAD^{10} or hu N-CAD^{20}, hu E-CAD^{10scr}, c E-CAD^{11flHAV} or c E-CAD^{HAVfl10}, peptides containing the HAV-flanking sequences of canine E-cadherin. A similar pattern of induction of invasion was obtained at 37°C with MDCK-*ras*-e c5 and with NMuMG derivatives expressing E-cadherin. In the latter tests, hu N-CAD^{10} and hu P-CAD^{10} did not significantly alter

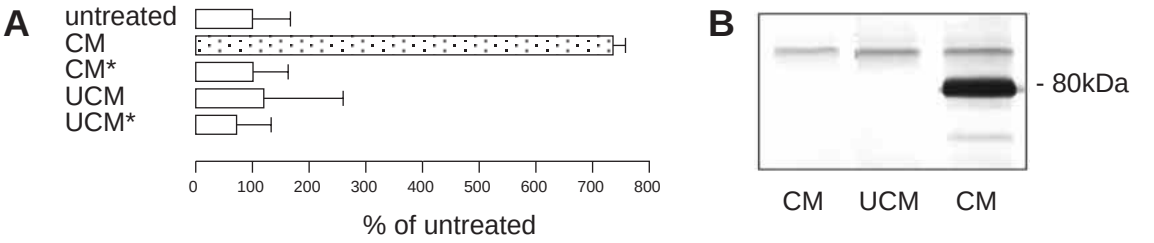

Fig. 3. Induction of invasion of MDCKts.*src*Cl2 cells into collagen type I by conditioned medium containing E-cadherin fragments (A). The cells were seeded on top of a collagen I gel and incubated at 40°C for 24 hours either untreated or in the presence of conditioned medium (CM), conditioned medium from which E-cadherin fragments were removed by immunoprecipition with DECMA-1 (CM*), unconditioned medium (UCM) or unconditioned medium on which a sham immunoprecipitation with DECMA-1 was performed (UCM*). The invasion index was calculated as the number of cells inside the gel over the total number of cells. Bars are the means as percentages of untreated cells; flags are standard deviations, and shaded bars indicate significant difference from untreated (P<0.005). Proteins immunoprecipitated by DECMA-1 from the conditioned medium (CM) or the unconditoned medium (UCM) were separated by SDS-PAGE, blotted and immunostained with DECMA-1 followed by goat anti-rat alkaline phosphatase conjugated antibody (lane 2 and 3) or with secondary antibody only (lane1) (B).

invasion. The non-cadherin HAV-containing decapeptides hu SOD^{10mut} and HA(HMIVF)^{10} were, like hu E-CAD^{10}, able to induce invasion in the E-cadherin-positive NMuMG derivatives. None of the decapeptides enhanced invasion of the constitutively invasive E-cadherin-negative NM-f-*ras*-TD1 cells (data not shown).

Also conditioned medium of MDCKts.*src*Cl2 cells, containing the 80 kDa fragment of E-cadherin, induced invasion of MDCKts.*src*Cl2 at 40°C. When cadherin fragments were removed by immunoprecipitation with DECMA-1, the conditioned medium did loose its ability to induce collagen type I invasion (Fig. 3).

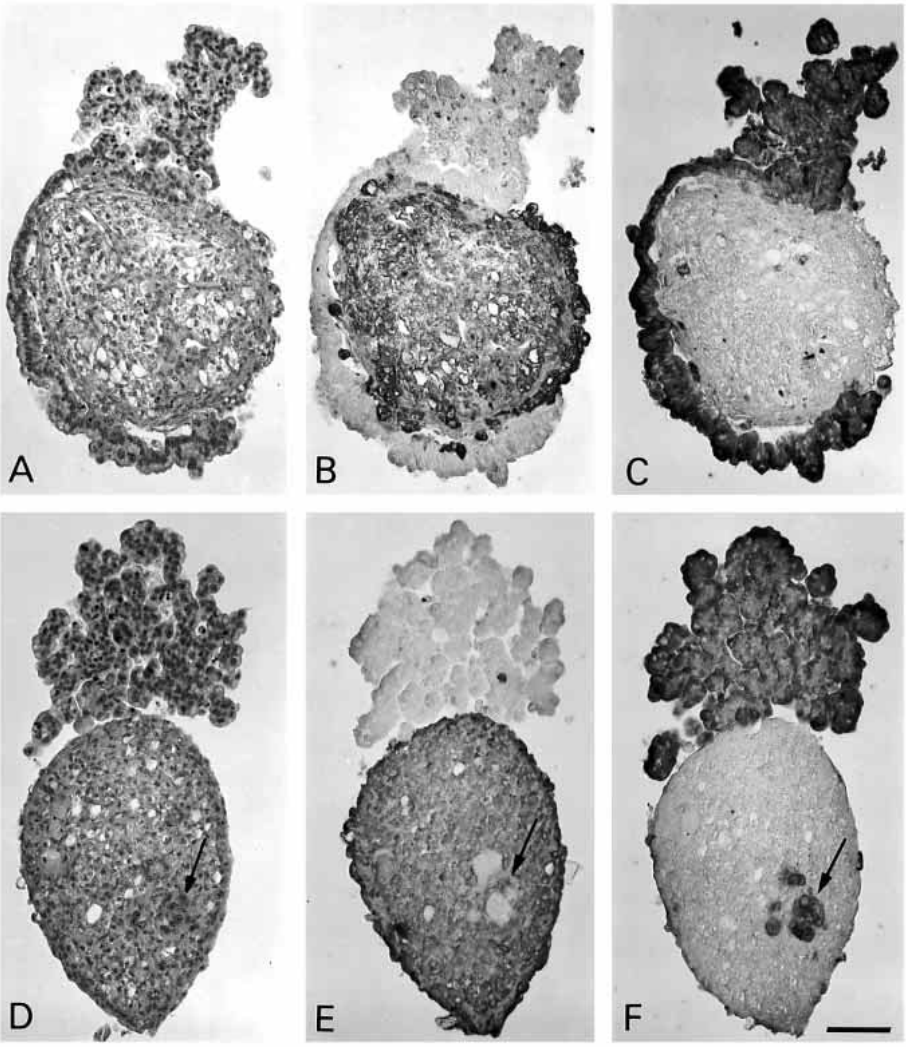

Fig. 4. Induction of invasion of MDCKts.*src*Cl2 at 40°C in chick heart fragments by hu E-CAD^{10} peptide. MDCKts.*src*Cl2 cells were confronted with 9-day-old precultured embryonic chick heart fragments on a semi-solid agar medium and after 24 hours transferred into a liquid medium and further cultured for 4 days. Confronting pairs were then fixed, embedded in paraffin and serially sectioned. Consecutive sections of confrontation cultures of MDCKts.*src*Cl2 cells, either untreated (A to C) or treated with hu E-CAD^{10} (D to F) were stained with hematoxyline-eosine (A and D), antiserum against chick heart (B and E) or with antiserum against MDCK cells (C and F). Arrows in D to F mark invasive MDCK cells. Bar, 50 μm.

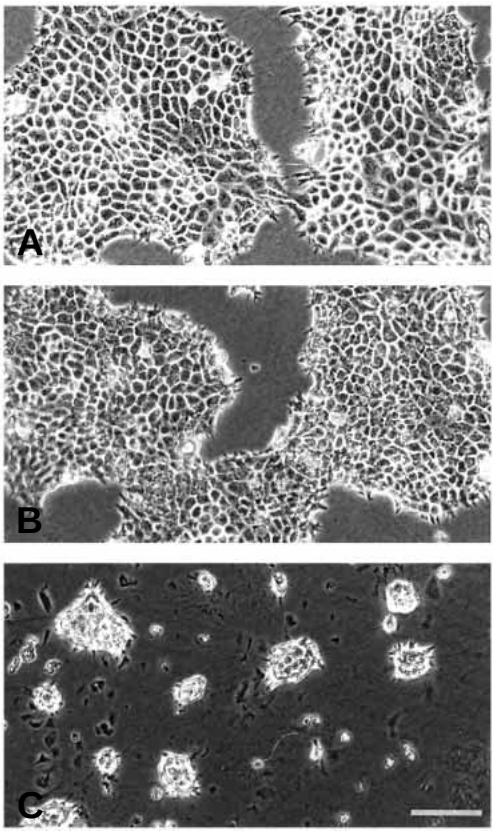

Fig. 5. Peptide c E-CAD^{20} disturbs the epithelioid morphotype of MDCKts.*src*Cl2 at 40°C on top of collagen I. MDCKts.*src*Cl2 cells were detached with trypsin/EDTA, seeded on top of a collagen I gel and incubated at 40°C. Photographs show non invading cells in focus on top of the gel after 9 days of culture either untreated (A) or treated with hu N-CAD^{20} (B) or c E-CAD^{20} peptide (C). Bar, 100 μm.

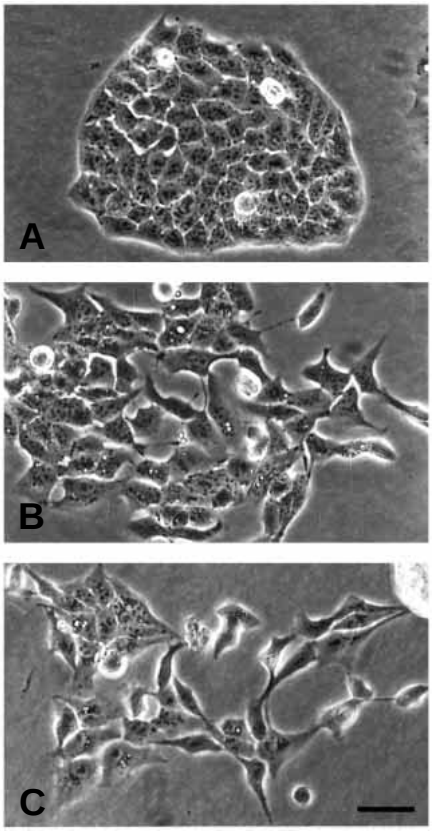

Fig. 6. Peptide hu E-CAD^{10} disturbs the epithelioid morphotype on tissue culture plastic substratum. MDCK-*ras*-e c5 were detached with trypsin/EDTA and seeded in a 24-well plate. When epithelioid islands were formed, the cells were either left untreated (A) or treated with hu E-CAD^{10} (B), DECMA-1 (C). Photographs were taken 4 hours later. Bar, 50 μm.

Invasion into chick heart

Three types of cells were tested also in the chick heart invasion assay. MDCKts.*src*Cl2 cells formed an epithelioid cell layer around the heart tissue after 4 days of confrontation at 40°C; invasion was not observed (Fig. 4A,B,C). Confrontation treatments with hu E-CAD^{10} (Fig. 4D,E,F) or with the DECMA-1 antibody (data not shown) perturbed the epithelioid organisation, and some MDCKts.*src*Cl2 cells were now found inside the heart tissue. Hu E-CAD^{10} did neither induce invasion nor interfere with the epithelioid organisation of NM-e-*ras* cells surrounding the precultured heart fragment. With NM-f-*ras*-TD-CAM5, not showing this epithelioid organisation despite their expression of exogenous murine E-cadherin, no induction of invasion by hu E-CAD^{10} or DECMA-1 was observed (data not shown).

Morphotype on solid substrata

The function of the E-cadherin/catenin complex is also reflected by the formation of epithelioid cell layers on solid substrata. Indeed, MDCKts.*src*Cl2 cells at 40°C formed large epithelioid colonies on top of collagen I (Fig. 5). In the presence of hu E-CAD^{10}, c E-CAD^{20} or DECMA-1, but not of hu N-CAD^{10} or hu N-CAD^{20}, the aggregates were much smaller and less compact.

Treatment of MDCK-*ras*-e c5 (Fig. 6) and MDCKts.*src*Cl2 cells cultured on tissue culture plastic substrate at 40°C with hu E-CAD^{10} or DECMA-1 did induce scattering.

DISCUSSION

HAV-containing peptides, with an amino acid sequence similar to the HAV-containing region of the first extracellular domain of cadherins, were shown to inhibit cadherin-dependent cellular functions. Using different cell lines, all expressing E-cadherin, and several assays we were able to demonstrate reproducible inhibition by HAV peptides homologous to E-cadherin. Our assays include morphotype on solid substrate, on collagen type I or in confronting organ culture with embryonic chick heart, the fast and slow aggregation as well as invasion into collagen type I or into precultured chick heart fragments. All these phenotypes were shown to be E-cadherin-dependent through inhibition with antibodies against E-cadherin.

The cadherin HAV peptides acted in a type- and species-specific manner (Tables 1 and 2). Peptides homologous to E-cadherin, but not peptides homologous to P- or N-cadherin, were able to block cadherin-related functions in cells expressing E-cadherin. The smaller degree of inhibition by hu

P-CAD[10] of the fast aggregation of MCF-7/AZ cells could be explained by the co-expression of P-cadherin on the surface of these cells. Similarly, the weak expression of N-cadherin on MDCKts.*src*Cl2 cells might explain the biphasic curve obtained by particle size counting after incubation of these cells with hu N-CAD[10]. Species-specificity is further illustrated by the lack of inhibition by hu E-CAD[10] of cadherin-related functions in cells expressing chicken E-cadherin. Both cadherin type-specific, and species-restricted activities suggest that homology in the HAV-flanking amino acids determines specificity. Limited differences in flanking amino acids between E-cadherin of different species, e.g. human versus murine or canine, do not interfere with the matching between the peptide and the cadherin expressed on the test cells.

We demonstrated an important role for the HAV sequence in the inhibition of E-cadherin functions, since peptides containing the HAV flanking amino acids but not the full HAV sequence and a scrambled peptide (see Table 1) were not active. Some HAV-containing peptides homologous to sequences of non-cadherin proteins showed E-cadherin inhibitory activity in the aggregation and collagen type I invasion assay despite large differences in HAV flanking sequences as compared to the cadherins expressed by the test cells. Such non-cadherin peptides did, however, underline also the role of the HAV sequence since HAA congeners were not active. Furthermore, they might reveal interactions between cadherins and other molecules expressed at the cell surface. Such interactions have been demonstrated already between N-cadherin and FGFR1 and FGFR2 (Williams et al., 1994). Another candidate for interactions with N-cadherin is ECSODB, since the ECSODB peptide inhibited N-cadherin-specific aggregation (Willems et al., 1995). These observations and our present experiments with the peptide homologous to the hemagglutinin of influenza virus (Stuart, 1994), suggest that other non-cadherin HAV-containing peptides and proteins might interfere with E-cadherin functions in an indirect way.

Quantitative and qualitative differences between the effects of HAV peptides and antibody against cadherin are obvious in our experiments. Depending upon the type of assay, the inhibition of E-cadherin dependent activities by DECMA-1 was stronger than by hu E-CAD[10]. Elongation of the peptides made them more potent inhibitors.

Our experiments with conditioned medium clearly show that not only synthetic E-cadherin peptides but also E-cadherin fragments, released in the medium, can induce invasion, provided that their concentration is relatively high. Cadherin proteins or peptides might be released to reach such a high concentration in the tumor microenvironment, e.g. by secretion or by proteolytic cleavage from the extracellular part of cadherin. Moreover, mutations resulting in a premature stop codon, as described in lobular breast carcinomas, may encode secretory forms of E-cadherin (Berx et al., 1996). Enhanced proteolysis has also been associated with invasion in experimental and in clinical cancer (Chambers and Matrisian, 1997). Proteases degrade not only the peritumoral matrix but may also cleave cell surface molecules. Such cleaved off peptides may compete with the native molecule for ligand binding as described for FGFR1 (Levi et al., 1996). Candidate E-cadherin degrading proteases are u-PA and MMP9. u-PA is upregulated in human breast cancer cells in culture after incubation with the E-cadherin antibody DECMA-1 (Frixen and Nagamine, 1993). MMP 9 is activated during invasion of the cytotrophoblast into the uterine wall and the activation is concomitant with an increased turn-over of E-cadherin, release of its 80 kDa fragments and replacement of E-cadherin by VE-cadherin (Zhou et al., 1997). The elevated levels of cadherin fragments in the urine and the plasma of cancer patients are compatible with an increased turn-over of the transmembrane E-cadherin protein (Katayama et al., 1994; Banks et al., 1995; Griffiths et al., 1996).

In conclusion, our experiments show that E-cadherin/catenin functions can be inhibited by homologous HAV-containing peptides and E-cadherin fragments. These or similar E-cadherin fragments may be released in an autocrine manner and in this way contribute to the maintenance of the invasive phenotype.

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REFERENCES

Banks, R. E., Porter, W. H., Whelan, P., Smith, P. H. and Selby, P. J. (1995). Soluble forms of the adhesion molecule E-cadherin in urine. *J. Clin. Pathol.* **48**, 179-180.

Becker, K. F., Atkinson, M. J., Reich, U., Huang, H. H., Nekarda, H., Siewert, J. R. and Höfler, H. (1993). Exon skipping in the E-cadherin gene transcript in metastatic human gastric carcinomas. *Hum. Mol. Genet.* **2**, 803-804.

Behrens, J., Mareel, M. M., Van Roy, F. M., and Birchmeier, W. (1989). Dissecting tumor cell invasion: epithelial cells acquire invasive properties following the loss of uvomorulin-mediated cell-cell adhesion. *J. Cell Biol.* **108**, 2435-2447.

Behrens, J., Vakaet, L., Friis, R., Winterhager, E., Van Roy, F., Mareel, M. M., and Birchmeier, W. (1993). Loss of epithelial morphotype and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/β-catenin complex in cells transformed with a temperature-sensitive *v-src* gene. *J. Cell Biol.* **120**, 757-766.

Berx, G., Cleton-Jansen, A.-M., Strumane, K., de Leeuw, W. J. F., Nollet, F., Van Roy, F. and Cornelisse, C. (1996). E-cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain. *Oncogene* **13**, 1919-1925.

Birchmeier, W., Weidner, K. M., Hülsken, J. and Behrens, J. (1993). Molecular mechanisms leading to cell junction (cadherin) deficiency in invasive carcinomas. *Semin. Cancer Biol.* **4**, 231-239.

Blaschuk, O. W., Sullivan, R., David, S. and Pouliot, Y. (1990). Identification of a cadherin cell adhesion recognition sequence. *Dev. Biol.* **139**, 227-229.

Bracke, M. E., Vyncke, B. M., Bruyneel, E. A., Vermeulen, S. J., De Bruyne, G. K., Van Larebeke, N. A., Vleminckx, K., Van Roy, F. M. and Mareel., M. M. (1993). Insulin-like growth factor I activates the invasion suppressor function of E-cadherin in MCF-7 human mammary carcinoma cells *in vitro*. *Br. J. Cancer* **68**, 282-289.

Bracke, M. E. and Mareel, M. M. (1994). Invasion assay using embryonic chick heart. In *Cell & Tissue Culture: Laboratory Procedures*, vol 5A:4 (ed. A. Doyle, J. B. Griffiths and D. G. Newell), pp. 1-16. John Wiley & Sons, Chicester.

Breviario, F., Caveda, L., Corada, M., Martin-Padura, I., Navarro, P., Golay, J., Introna, M., Gulino, D., Lampugnani, M. G. and Dejana, E. (1995). Functional properties of human vascular endothelial cadherin (7B4/cadherin-5), an endothelium-specific cadherin. *Arterioscler. Thromb. Vasc. Biol.* **15**, 1229-1239.

Brieher, W. M., Yap, A. S. and Gumbiner, B. M. (1996). Lateral dimerization is required for the homophilic binding activity of C-cadherin. *J. Cell Biol.* **135**, 487-496.

Byers, S., Amaya, E., Munro, S. and Blaschuk, O. (1992). Fibroblast growth factor receptors contain a conserved HAV region common to cadherins and influenza strain A hemagglutinins: a role in protein-protein interactions? *Dev. Biol.* **152**, 411-414.

Chambers, A. F. and Matrisian, L. M. (1997). Changing views of the role of matrix metalloproteinases in metastasis. *J. Nat. Cancer Inst.* **89**, 1260-1270.

Frixen, U. H. and Nagamine, Y. (1993). Stimulation of urokinase-type plasminogen activator expression by blockage of E-cadherin-dependent cell-cell adhesion. *Cancer Res.* **53**, 3618-3623.

Griffiths, T. R. L., Brotherick, I., Bishop, R. I., White, M. D., McKenna, D. M., D. M. Horne, D. M., Shenton, B. K., Neal, D. E. and Mellon, J. K. (1996). Cell adhesion molecules in bladder cancer: soluble serum E-cadherin correlates with predictors of recurrence. *Br. J. Cancer* **74**, 579-584.

Hamaguchi, M., Matsuyoshi, N., Ohnishi, Y., Gotoh, B., Takeichi, M. and Nagai, Y. (1993). p60*v-src* causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. *EMBO J.* **12**, 307-314.

Herrenknecht, K. and Kemler, R. (1993). Characterization of recombinant E-cadherin (uvomorulin) expressed in insect cells. *J. Cell Sci. Suppl. 17*, 147-154.

Hinck, L., Näthke, I. S., Papkoff, J. and Nelson, W. J. (1994). Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly. *J. Cell Biol.* **125**, 1327-1340.

Hyafil, F., Morello, D., Babinet, C. and Jacob, F. (1980). A cell surface glycoprotein involved in the compaction of embryonal carcinoma cells and cleavage stage embryos. *Cell* **21**, 927-934.

Katayama, M., Hirai, S., Kamihagi, K., Nakagawa, K., Yasumoto, M. and Kato, I. (1994). Soluble E-cadherin fragments increased in circulation of cancer patients. *Br. J. Cancer* **69**, 580-585.

Koch, A. W., Pokutta, S., Lustig, A. and Engel, J. (1997). Calcium binding and homoassociation of E-cadherin domains. *Biochemistry* **36**, 7697-7705.

Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Levi, E., R. Fridman, H.-Q. Miao, Y.-S. Ma, A. Yayon, and I. Vlodavsky. 1996. Matrix metalloproteinase 2 releases active soluble ectodomain of fibroblast growth factor receptor 1. *Proc. Nat. Acad. Sci. USA* **93**, 7069-7074.

Mareel, M., Kint, J. and Meyvisch, C. (1979). Methods of study of the invasion of malignant C3H mouse fibroblasts into embryonic chick heart *in vitro*. *Virchows Arch. B Cell Pathol.* **30**, 95-111.

Mareel, M., Bracke, M. and Van Roy, F. (1994). Invasion promoter versus invasion suppressor molecules: the paradigm of E-cadherin. *Mol. Biol. Rep.* **19**, 45-67.

Matsuyoshi, N., Hamaguchi, M., Tanigushi, S., Nagafuchi, A., Tsukita, S. and Takeichi, M. (1992). Cadherin-mediated cell-cell adhesion is perturbed by *v-src* tyrosine phosphorylation in metastatic fibroblasts. *J. Cell Biol.* **118**, 703-714.

Mbalaviele, G., Chen, H., Boyce, B. F., Mundy, G. R. and Yoneda, T. (1995). The role of cadherin in the generation of multinucleated osteoclasts from mononuclear precursors in murine marrow. *J. Clin. Invest.* **95**, 2757-2765.

Mège, R. M., Matsuzaki, F., Gallin, W. J., Goldberg, J. I., Cunningham, B. A. and Edelman, G. M. (1988). Construction of epithelioid sheets by transfection of mouse sarcoma cells with cDNAs for chicken cell adhesion molecules. *Proc. Nat. Acad. Sci. USA* **85**, 7274-7278.

Mège, R. M., Goudou, D., Diaz, C., Nicolet, M., Garcia, L., Geraud, G. and Rieger F. (1992). N-cadherin and N-CAM in myoblast fusion: compared localisation and effect of blockade by peptides and antibodies. *J. Cell Sci.* **103**, 897-906.

Nagar, B., Overduin, M., Ikura, M. and Rini, J. M. (1996). Structural basis of calcium-induced E-cadherin rigidification and dimerization. *Nature* **380**, 360-364.

Näthke, I. S., Hinck, L., Swedlow, J. R., Papkoff, J. and Nelson, W. J. (1994). Defining interactions and distributions of cadherin and catenin complexes in polarized epithelial cells. *J. Cell Biol.* **125**, 1341-1352.

Overduin, M., Harvey, T. S., Bagby, S., Tong, K. I., Yau, P., Takeichi, M. and Ikura, M. (1995). Solution structure of the epithelial cadherin domain responsible for selective cell adhesion. *Science* **267**, 386-389.

Pokutta, S., Herrenknecht, K., Kemler, R. and Engel, J. (1994). Conformational changes of the recombinant extracellular domain of E-cadherin upon calcium binding. *Eur. J. Biochem.* **223**, 1019-1026.

Shapiro, L., Fannon, A. M., Kwong, P. D., Thompson, A., Lehmann, M. S., Grübel, G., Legrand, J. F., Als-Nielsen, J., Colman, D. R. and Hendrickson, W. A. (1995). Structural basis of cell-cell adhesion by cadherins. *Nature* **374**, 327-337.

Stuart, D. (1994). Docking mission accomplished. *Nature* **371**, 19-20.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* **251**, 1451-1455.

Takeichi, M. (1993). Cadherins in cancer: implications for invasion and metastasis. *Curr. Opin. Cell Biol.* **5**, 806-811.

Takeichi, M. (1995). Morphogenetic roles of classic cadherins. *Curr. Opin. Cell Biol.* **7**, 619-627.

Vakaet, L. Jr, Vleminckx, K., Van Roy, F. and Mareel, M. (1991). Numerical evaluation of the invasion of closely related cell lines into collagen type I gels. *Invasion Metast.* **11**, 249-260.

Vandenbossche, G. M. R., De Bruyne, G. K., Bruyneel, E. A., Clemminck, G., Vleminckx, K., Van Roy, F. M. and Mareel, M. M. (1994). Microencapsulation of MDCK-*ras*-e cells prevents loss of the E-cadherin invasion suppressor function *in vivo*. *Int. J. Cancer* **57**, 73-80.

Vermeulen, S. J., Bruyneel, E. A., Van Roy, F. M., Mareel, M. M. and Bracke, M. E. (1995). Activation of the E-cadherin/catenin complex in human MCF-7 breast cancer cells by all-trans-retinoic acid. *Br. J. Cancer* **72**, 1447-1453.

Vleminckx, K., Vakaet, L. Jr, Mareel, M., Fiers, W. and Van Roy, F. (1991). Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell* **66**, 107-119.

Wheelock, M. J., Buck, C. A., Bechtol, K. B. and Damsky, C. H. (1987). Soluble 80-kd fragment of cell-CAM 120/80 disrupts cell-cell adhesion. *J. Cell. Biochem.* **34**, 187-202.

Willems, J., Zwijsen, A., Slegers, H., Nicolaï, S., Bettadapura, J., Raymackers, J. and Scarcez, T. (1993). Purification and sequence of rat extracellular superoxide dismutase B secreted by C6 glioma. *J. Biol. Chem.* **268**, 24614-24621.

Willems, J., Bruyneel, E., Noë, V., Slegers, H., Zwijsen, A., Mège, R.-M. and Mareel, M. (1995). Cadherin-dependent cell aggregation is affected by decapeptide derived from rat extracellular super-oxide dismutase. *FEBS Lett.* **363**, 289-292.

Williams, E., Furness, J., Walsh, F. S. and Doherty, P. (1994). Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, NCAM and N-cadherin. *Neuron* **13**, 583-594.

Zhou, Y., Fisher, S. J., Janatpour, M., Genbacev, O., Dejana, E., Wheelock, M. and Damsky, C. H. (1997). Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? *J. Clin. Invest.* **99**, 2139-2151.