Role antitumor lipids in cell-cell adhesion and invasion
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The alkyl-lysophospholipid 1-<i>O</i>-octadecyl-2-<i>O</i>-methyl-glycerophosphocholine induces invasion through episialin-mediated neutralization of E-cadherin in human mammary MCF-7 cells <i>in vitro</i>.

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Abstract

l-O-octadecyl-2-O-methyl-glycerophosphocholine (ET-18-OMe) is an analogue of the naturally occurring 2-lysophosphatidylcholine belonging to the class of antitumor lipids. Previously, we demonstrated that ET-18-OMe modulates cell-cell adhesion of human breast cancer MCF-7 cells. In the present study we tested the effect of ET-18-OMe on adhesion, invasion and on localisation of episialin and E-cadherin in MCF-7/AZ cells, expressing a functional E-cadherin/catenin complex. The MCF-7/6 human breast cancer cells were used as negative control since their E-cadherin/catenin complex is functional in cells grown on solid substrate but not in suspension. The function of E-cadherin, a calcium-dependent transmembrane cell-cell adhesion and signaltransducing molecule, is disturbed in invasive cancers by mutation, loss of mRNA stability, proteolytic degradation, tyrosine phosphorylation of associated proteins and large cell-associated proteoglycans or mucin-like molecules such as episialin. Episialin, also called MUC1, is an anti-adhesion molecule which by its large number of glycosylated tandem repeats can sterically hinder the adhesive properties of other glycoproteins.

ET-18-OMe inhibited the E-cadherin functions of MCF-7/AZ cells as measured by inhibition of fast and slow aggregation and by the induction of collagen invasion: these effects were enhanced by MB2, an antibody against E-cadherin and blocked by monoclonal antibodies 214D4 or M8, against episialin. Transcription, translation, protein turnover and cell surface localisation of episialin were not altered. ET-18-OMe induced finger-like extensions with clustering of episialin together with E-cadherin and carcinoembryonic antigen, but not with occludin. In cells in suspension, ET-18-OMe caused a shift in the flow-cytometric profile of episialin towards a lower intensity for MCF-7/AZ cells.

In contrast with MCF-7/AZ cells, the adhesion-deficient and non-invasive MCF-7/6 cells showed neither morphotypic changes nor induction of aggregation nor invasion in collagen I upon treatment with ET-18-OMe. Co-localisation of episialin with E-cadherin was rarely observed.

We conclude that in the human breast cancer cells MCF-7/AZ, E-cadherin and episialin are key molecular players in the regulation of promotion and suppression of cell-cell adhesion and invasion.
Introduction

1-O-octadecyl-2-O-methyl-glycerophosphocholine (ET-18-OMe) interacts with cell membranes, thereby modulating phospholipid metabolism, interfering with signal transduction, inducing apoptosis and differentiation, and regulating invasion (Lohmeyer and Bittman, 1994). On solid substrate, the E-cadherin/catenin complex is implicated in the cell-cell adhesion of two variants of the MCF-7 human breast cancer cell line family, coined MCF-7/AZ and MCF-7/6. In suspension, however, MCF-7/6 cells are adhesion-deficient while MCF-7/7AZ cells are adhesion-proficient. Treatment with ET-18-OMe caused antithetic effects on the two types of MCF-7 cells. ET-18-OMe restored the E-cadherin function in the adhesion-deficient MCF-7/6 cells, whereas it caused a decrease in cellular aggregation of the adhesion-proficient MCF-7/7AZ cells. The latter effects could not be explained by changes in sialylation of E-cadherin (Steelant et al., 1999). Antithetic effects on invasion have also been reported: ET-18-OMe inhibited invasion of constitutively invasive cells (Bolscher et al., 1988; Engebraaten et al., 1991; Slaton et al., 1994; Haugland et al., 1999) whereas it induced invasion of otherwise non-invasive cells (Bolscher et al., 1986; Bruyneel et al., 1989). Inhibition of invasion in the presence of ET-18-OMe was explained by induction of host tissue resistance (Schallier et al., 1991) but the antithetic effects on cell-cell adhesion and the invasion promoting effect of ET-18-OMe have not been explained.

Adhesion and invasion are the result of a balance between promotor and suppressor molecules modulated by multiple intra- and extracellular factors. The presence of the anti-adhesion molecule episialin on the two types of MCF-7 cells was observed in a previous study (Steelant et al., 1999). The aim of the present study was to see whether ET-18-OMe affects invasion and adhesion through modulation of episialin and E-cadherin.

Episialin is a heterodimeric molecule composed of a membrane anchor and an extracellular moiety. The membrane anchor consists of a cytoplasmatic domain of 68 amino acids and a transmembrane domain. The extracellular moiety consists of a large mucin domain containing a large number of O-linked glycans (Hilkens et al., 1995a). The carbohydrate side chains carry many sialic acid residues conveying a highly negative charge upon episialin (Hilkens and Buijs, 1988). Episialin is located at the apical surface of most glandular epithelial cells; it is overexpressed in carcinomas, often distributed over the entire cell surface (Hilkens and Buijs, 1988). Overexpression of episialin inhibits integrin-mediated cell adhesion to the extracellular matrix (Wesseling et al., 1995), suppresses cellular aggregation (Ligtenberg et al., 1992; Hilkens et al., 1995b), and increases invasiveness (Hilkens et al., 1995b). Episialin can be released from the cell surface by an unknown mechanism. In patients with breast and other carcinomas, shed episialin from the cancer cells can be detected in the circulation by the CA 15-3 assay, which is presently used for monitoring the course of breast cancer and for early detection of recurrent disease (Hilkens, 1992).

The cell-cell adhesion molecule, E-cadherin, linked by its cytoplasmic part via β-catenin, or plakoglobin and α-catenin to the actin cytoskeleton, acts as an invasion-suppressor (Bracke et al., 1996). The E-cadherin/catenin complex is often disturbed in cancer cells at various
levels, namely by mutations in the cadherin or catenin genes, reduced stability of mRNAs, tyrosine phosphorylation of β-catenin, intra- and extracellular associations with other proteins, steric hindrance of enlarged proteoglycans and by ectodomain shedding of HAV (Histidine Alanine Valine)-containing E-cadherin-specific peptides. Inactivation of the E-cadherin/catenin complex leads to increased invasiveness as documented in experimental as well as clinical cancers [Bracke et al., 1991; Mareel et al., 1994; Behrens et al., 1994; Takeichi, 1995; Bracke et al., 1996; Mareel and Van Roy, 1998; Noë et al., 1999].

In the adhesion-proficient MCF-7/AZ and adhesion-deficient MCF-7/6 human breast cancer cell lines, the E-cadherin/catenin complex (Bracke et al., 1991) and the anti-adhesion molecule episialin, are both expressed (Remingbald et al., 1996). Altered expression of episialin is reported to interfere with cellular aggregation (Ligtenberg et al., 1992; Kondo et al., 1998). We therefore investigated the influence of ET-18-OMe on the expression and localisation of episialin as well as its influence on the structure and function of the E-cadherin/catenin complex.

Materials and Methods

MCF-7 cell variants
MCF-7/AZ and MCF-7/6 cells are variants of the human mammary carcinoma cell family MCF-7 (Bracke et al., 1991). The cells are maintained on tissue culture plastic substrate (Falcon, Becton Dickinson, Aalst, Belgium) in a mixture of Dulbecco's Modified Eagle's Medium (DMEM) and HAM F12 (50/50) (Gibco, Paisley, Scotland) supplemented with 0.05% (w/v) glutamine, 250 IU/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum. MCF-7/AZ differ from MCF-7/6 cells in that they are not invasive in vitro when tested in the precultured chick heart invasion assay (Bracke et al., 1991) or in the matrigel chemoinvasion assay (Simon et al., 1992).

ET-18-OMe
ET-18-OMe (clinical grade) was kindly provided by Dr. P. Hilgard (ASTA MEDICA, Frankfurt am Main, Germany). Cells were cultured in the presence of 25 μg ET-18-OMe/ml culture medium for 48 h and the drug remained present during the subsequent assays (Steelant et al., 1999). To measure cellular uptake, [3H]-ET-18-OMe (specific activity: 58 Ci/mol; 37 mBq/ml) was custom made at the Laboratory for Organic and Bio-Organic Synthesis (Ghent University, Belgium) in collaboration with Amersham Corp. (Arlington Heights, IL). Cells were treated with 25 μg ET-18-OMe/ml containing (0.04 μCi/μg/ml) [3H]-ET-18-OMe for 48 h, washed three times, subsequently lysed with PBS containing 1% Triton X-100, 1% NP-40 and processed for scintillation counting in a LS 3801 (Beckman, California, USA). Drug toxicity was evaluated through measurement of mitochondrial dehydrogenase activities with an MTT-reagent (Sigma) as described (Romijn et al., 1988).
Antibodies
HECD-1 (Takara Shuzo Co., Kyoto, Japan) and α-Umt (Behrens et al., 1989) are mouse monoclonal and rabbit polyclonal antibodies used to reveal respectively human and dog E-cadherin. The mouse monoclonal antibody MB2 was raised against MCF-7/AZ cells and selected for functional inactivation of E-cadherin (Bracke et al., 1993). M8 (a gift from Dr. D.M. Swallow, MRC, Human Biochemical Genetics Unit, London, UK) and 214D4 (Wesseling et al., 1995) are mouse monoclonal antibodies recognizing the peptide backbone of human epsialin. Rabbit polyclonal antibodies against β-catenin or against α-catenin and phalloidin-FITC were from Sigma (St. Louis, MO). The secondary antibodies sheep anti-mouse labeled with FITC, and the Texas Red-labeled donkey anti-rabbit, sheep anti-mouse and sheep anti-rat were obtained from Amersham (Gent, Belgium), FITC-pig anti-rabbit from Dako (Denmark). Treatment with neutralizing antibodies was during the functional assay, except for the fast aggregation assay where cells were pretreated with the antibodies for 30 min at 4°C.

Collagen type I invasion
6-well plates were filled with 1.25 ml of neutralized type I collagen (0.09%;w/v)(Upstate Biotechnology, Lake Placid, NY) and incubated for at least 1 h at 37°C to allow gelification. Single-cell suspensions, detached from their plastic tissue culture substrate with trypsin/EDTA (0.25% trypsin, 0.02% EDTA, 0.9% NaCl), were seeded on top of the collagen gel and cultures were incubated at 37°C for 24 h. Using an inverted microscope controlled by a computer program, we counted the invasive and superficial cells in 12 fields of 0.157 mm². The invasion index expresses the percentage of cells invading the gel over the total number of cells (Bracke et al., 1999).

Cellular aggregation
Aggregation assays were as described previously (Bracke et al., 1991, Boterberg et al., 1999). For the slow aggregation assay, single cell suspensions, prepared as for the collagen invasion assay, were seeded on top of a semi-solid agar medium. After 24 h, aggregate formation was evaluated subjectively under an inverted phase contrast microscope (Leitz, Wetzlar, Germany) at magnification x 40. For the fast aggregation assay, single cell suspensions were prepared in accordance with an E-cadherin-saving procedure described by Bracke et al. (1993). Cells were incubated in an isotonic buffer containing 1.25 mM Ca²⁺ under Gyrotory shaking (New Brunswick Scientific, New Brunswick, NJ) at 80 rpm for 30 min. Particle diameters were measured in a Coulter particle size counter LS 200 (Coulter, Lake Placid, NY) at the start (N₀) and after 30 min of incubation (N₃₀) and plotted against percentage volume distribution.

Radioimmuno-assay of epsialin
Epsialin associated with the cells or shed into the culture medium was measured with an assay based on the measurement of the CA 15.3 antigen (Hilkens, 1992) as described in the protocol of DPC (Diagnostic Products Corporation, Los Angeles, CA). Briefly, ligand coated tubes and two murine monoclonal anti-CA 15.3 antibodies where used, one [¹²⁵I]-labeled and the other ligand-labeled. The epsialin in the culture medium or in total cell lysates was captured
between the monoclonals, in a reaction proceeding with liquid-phase kinetics. Separation was then achieved by the ligand-coated tube/anti-ligand bridge method and then $^{125}$I was counted in a gamma counter and expressed as units episialin/mg protein.

**Northern-blot analysis of episialin**

Northern-blot analysis of episialin mRNA was performed with a method developed to analyse large mRNAs (Debailleul et al., 1998). Briefly, RNA samples were denatured and then fractionated by electrophoresis through a 0.9% agarose denaturating gel, followed by transfer onto Hybond$^{TM}$-N$^+$ membrane. The Episialin and GAPDH probe (Gendler et al., 1990) were labeled with $^{32}$P dCTP using a commercial random-primed labeling kit (Boehringer Mannheim, Mannheim, Germany) in accordance with the manufacturer's protocol. The membrane was exposed to Kodak X-Omat film at $-80^\circ$C.

**Immunoprecipitation of episialin**

For immunoprecipitation of episialin, confluent cultures were washed three times with phosphate-buffered saline (PBS) and lysed with PBS containing 1% Triton X-100, 1% NP-40, 300 µg/ml PMSF, 10 µg/ml leupeptin and 10 µg/ml aprotinin. 100 µl beads coated with anti-mouse/rat IgG (SAC-CEL, IDS, Boldon, UK) were preincubated with 200 µl monoclonal antibody 214D4 in PBS with 1% Triton X-100 and 1% NP-40 at 4° C for 1 h before addition of lysate and further incubation for 2 h. Then, beads were washed three times with lysis buffer and the proteins were eluted with Laemmli buffer (Laemmli, 1970) and processed for Western-blotting.

**Biotinylation of cell surface proteins**

To investigate internalisation of episialin, cells were biotinylated, in accordance with Litvinov and Hilkens (1993). Briefly, cultures were incubated with 100 µg N-hydroxydisulfosuccimide-biotin (NHS-SS-biotin, Pierce, Chemical Co., Rockford, IL) per ml for 30 min at 0°C and recovery of the biotinylated proteins was with avidin-agarose beads (50%) (Pierce) for Western-blotting. To investigate internalisation of E-cadherin, cells were biotinylated as described by Boterberg et al. (2000) and processed for Western-blotting.

**Western blotting of episialin and E-cadherin**

Equal amounts of protein (50 µg) were loaded for sodiumdodecylsulfate polyacrylamide gradient (4-20%) gel electrophoresis under reducing conditions. Electroblotting on nitrocellulose membranes (Biotrace NT, Gelman Science, Ann Arbor, MI) was in accordance with standard procedures (Glas et al., 1981). Each blot was then treated with 5% Gloria milk (Nestlé, Vevey, Switzerland) in tris-buffered saline (TBS) for 4 h prior to overnight incubation with monoclonal antibody 214D4 for Episialin or HEC-D-1 for E-cadherin. After washing, blots were incubated for 2 h with horseradish peroxidase conjugated secondary antibody. Proteins were stained using ECL reagent (Amersham) as a substrate.
Immunofluorescence detection of episialin and the E-cadherin/catenin complex.

Cells, brought in suspension by the abovementioned E-cadherin-saving procedure, were stained by an indirect immunofluorescence technique. To this end, cells were incubated at 4°C for 1 h with 214D4 as the primary antibody against episialin or with HECD-1 against E-cadherin, followed by washing with Ca, Mg-free-Hanks’ buffered salt solution (CMF-HBSS) and incubation in the dark at 4°C for 1 h with an FITC-labeled sheep anti-mouse secondary antibody. Then, cells were washed, fixed with 3% paraformaldehyde in PBS at room temperature for 20 min and analyzed by flow cytometry with a FACSort (Becton Dickinson, Mountain view, CA).

Cells, established on glass coverslips, were fixed in the same way and incubated with 214D4 at room temperature for 1 h, followed by washing and incubation with the secondary antibody for another hour and examination under the fluorescence microscope (Dialux,Leitz). For confocal laser scanning with a Leica DM IRBE fluorescence microscope and a Leica TCS NT confocal unit, the cells were fixed for 20 min followed by incubation in 50 mM NH₄Cl in PBS, permeabilisation with 0.2% Triton X-100 for 5 min, washing with PBS and quenching with 5% BSA in TBS for 30 min, all at room temperature. Incubation with primary antibodies was for 1 h, followed by washing and incubation both with FITC-conjugated and Texas Red-conjugated secondary antibodies as indicated in the Results section.

Statistics

All treatments were matched and carried out at least two times. The student t-test (95%) and the F-test (95%) were used for statistical analysis of the collagen invasion assay, radioimmunoassay, and uptake measurements of [³H]-ET-18-OME. The Kolmogorov-Smirnov statistics were used to analyze the differences between the volume distribution curves in the fast aggregation assay and for flow cytometry. Northern-blot of episialin, immunoblots of episialin, E-cadherin, β-catenin or α-catenin were quantified with statistical software quantiscan v2.0 (Biosoft, UK).

Results

Viability and uptake of ET-18-OMe

MCF-7/AZ or MCF-7/6 cells treated with 25 μg ET-18-OMe/ml in presence of serum for 48h had a viability of 80% as compared to untreated cultures, in line with previous observations (Steelant et al., 1999). Under these circumstances, the uptake of [³H]-ET-18-OMe was 3.6 +/- 0.1 μg/mg protein for MCF-7/AZ cells and 3.4 +/- 0.2 μg/mg protein for MCF-7/6 cells.

Effect of ET-18-OMe on E-cadherin- and episialin-related phenotypes

Invasion into collagen type I

Neither MCF-7/AZ nor MCF-7/6 cells (data not shown) invaded into collagen (Figure 1). Invasion of MCF-7/AZ cells was induced by the anti-E-cadherin antibody MB2. Monoclonal antibodies 214D4 or M8 against episialin had no significant effect on untreated non-invasive cells, nor on MB2-induced invasion. ET-18-OMe treatment induced invasion of MCF-7/AZ cells and this was enhanced by MB2 antibody. ET-18-OMe-induced invasion was prevented.
by 214D4 or M8 antibodies, and this prevention was neutralized whenever MB2 antibody was present. MCF-7/6 cells, manipulated with antibodies in the same way and treated or not with ET-18-OMe invariably failed to invade into collagen type I with invasion indices all between 0% and 1.5% (data not shown).

**Figure 1**: Effect of antibodies against E-cadherin (MB2) or against episialin (214D4 or M8) on invasion into collagen type I of MCF-7/AZ cells treated (full bars) or not (open bars) with ET-18-OMe. Bars and flags indicate mean values and standard deviations, respectively; asterisks indicate statistical difference from control (MCF-7/AZ cells without antibodies).

**Cellular aggregation**

MCF-7/AZ cells formed large and compact aggregates (Figure 3A), while the MCF-7/6 cells formed numerous small and irregular dispersed aggregates (Figure 3G). Spontaneous aggregation of MCF-7/AZ cells (Figure 3A) was inhibited by ET-18-OMe (Figure 3C), as well as by MB2 (Figure 3B) or the combination of ET-18-OMe and MB2 (Figure 3D), with the formation of numerous small and dispersed aggregates. The ET-18-OMe-mediated inhibition of aggregation was counteracted by 214D4 (Figure 3E) or M8 antibodies (data not shown) leading to formation of small aggregates. Addition of MB2 antibody neutralised the action of the antibodies against episialin (Figure 3F) with cells dispersed over the substratum. ET-18-OMe induced large and compacted aggregates of MCF-7/6 cells (Figure 3I) which were altered to more dispersed aggregates by 214D4 (Figure 3K) or M8 (data not shown). MB2 (Figure 3J) or the combination of MB2 with 214D4 (Figure 3L) or MB2 with M8 (data not shown) prohibited the aggregation.
Figure 2: Slow aggregation assay for 24 h on semi-solid agar medium of MCF-7 cell variants, treated with ET-18-OMe (+, panels C to F and I to L) or not (-, panels A, B, G and H); aggregation was done without (none) or with antibodies against E-cadherin (MB2) and/or against episialin (214D4) as indicated.
Scale bar = 0.5 mm.
In the fast aggregation assay, cell-cell adhesion of MCF-7/AZ cells was inhibited by ET-18-OMe, as well as by MB2 (Figure 3A). The ET-18-OMe-mediated inhibition was counteracted by 214D4 or M8 antibodies (Figure 3B). Addition of MB2 antibody neutralized ET-18-OMe-induced aggregation and the action of the antibodies against episialin. In contrast, ET-18-OMe induced aggregation of MCF-7/6 cells and this process was not altered by 214D4 or M8. MB2 or the combination of MB2 with 214D4 or M8 prevented aggregation (Figure 3C).

Effect of ET-18-OMe on the expression of episialin and E-cadherin

Radioimmuno-assay of episialin

No significant differences could be observed in the amount of cell-associated or released episialin between MCF-7/AZ and MCF-7/6 cells nor between ET-18-OMe treated and untreated cultures (Table 1).

Table 1: Radioimmuno-assay detection of episialin, expressed as units episialin/mg protein.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treatment with ET-18-OMe</th>
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<tbody>
<tr>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>cells</td>
</tr>
<tr>
<td>MCF-7/AZ</td>
<td>2.1 +/- 0.3</td>
</tr>
<tr>
<td>MCF-7/6</td>
<td>2.0 +/- 0.1</td>
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Episialin mRNA

Northern-blot analysis of MCF-7/AZ and MCF-7/6 cells demonstrated a mRNA at 4.4-kb and a second larger mRNA at 7.1-kb (Figure 4), in line with the known polymorphism that is characteristic of the mucin genes as demonstrated for MCF-7 cells by others (Inatoma et al., 1995; Debaileul et al., 1998). Treatment with ET-18-OMe did not significantly alter the episialin mRNA levels.

Figure 4: Northern-blot analysis of episialin in MCF-7/AZ and MCF-7/6 cells treated or not with ET-18-OMe. Both MCF-7 variants have a 4.4-kb mRNA and a second 7.1-kb mRNA. The same amount of mRNA was loaded as evidenced by GAPDH.

Immunoprecipitation, biotinylation and Western blotting

Immunoprecipitation of total episialin without (data not shown) or with biotinylation (Figure...
5A), followed by Western-blotting revealed two distinct proteins of above 200 kDa, resulting from the co-dominant expression of the two episialin mRNAs shown by the Northern-blot. Treatment with ET-18-OMe combined or not with 214D4 had no effect on the expression level of the episialin protein doublet. Biotinylation and Western-blotting carried out with HECD-1 revealed a 120 kDa band representing E-cadherin at the cell surface (Figure 5B). Treatment with ET-18-OMe or ET-18-OMe combined with 214D4 did not significantly alter the expression level of the episialin protein doublet nor of E-cadherin, indicating their unaltered expression at the cell surface.

Figure 5: (A) Immunoblot of biotinylated episialin of MCF-7/AZ and MCF-7/6 cells, treated or not with ET-18-OMe or with ET-18-OMe + 214D4 as described in materials and methods. The episialin protein doublet was observed > 200 kDa range. (B) Immunoblot of biotinylated E-cadherin of MCF-7/AZ and MCF-7/6 cells, treated or not with ET-18-OMe or with ET-18-OMe + 214D4 as described in materials and methods. The use of HECD-1 revealed a 120 kDa band representing E-cadherin.

Confocal Laser Scanning Microscopy In cells on solid substrate, co-localisation of β-catenin, α-catenin and actin with E-cadherin was observed for both MCF-7/AZ and MCF-7/6 cells (data not shown) in line with earlier observations (Boterberg et al., 2000). Episialin and E-cadherin were found at the apical and at the lateral aspect of the plasmamembrane, respectively, without co-localisation (Figure 6A). Treatment of MCF-7/AZ cells with ET-18-OMe caused a more irregular pattern of the E-cadherin/catenin complex but co-localisation of the elements of the complex was maintained (data not shown). Strikingly, ET-18-OMe induced clustering of episialin (Figure 6B1) and E-cadherin (Figure 6B2) in finger-like extensions and co-localisation of both molecules (Figures 6B3): this was strongly reduced by incubation with 214D4 for 24 h although clustering and co-localising with E-cadherin could occasionally be observed (Figures 6C3). Finger-like extensions, clustering of episialin and co-localisation with E-cadherin was rarely observed for MCF-7/6 cells (data not shown).
Figure 6: Confocal laser scanning microscopy of MCF-7/AZ (panels A, B, and C) cells seeded on solid substrate, untreated (panels A) or treated with ET-18-OMe (panels B and C) in the presence of antibody 214D4 against episialin (panel C) or not (panel B) and immunocytochemically double stained with antibodies 214D4 against episialin and α-UTM against E-cadherin. Green FITC fluorescence was used for episialin (panels A1, B1 and C1), Texas red fluorescence for E-cadherin (panels A2, B2 and C2). A yellow color (examples indicated by arrow heads) shows co-localisation of episialin and E-cadherin (panels A3, B3 and C3). Scale bar = 10 mm.
Flow cytometric analysis of episialin/E-cadherin

Flow cytometry of unpermeabilized MCF-7 cells in suspension revealed an episialin-specific fluorescent signal in the MCF-7/AZ cells with a peak at a higher intensity and less heterogeneity than in MCF-7/6 cells (Figure 7). Treatment of MCF-7/AZ cells with ET-18-OMe caused a shift of the median fluorescence signal toward a lower intensity (Figure 7B). Treatment of MCF-7/6 cells with ET-18-OMe did not change the peak but the profile was different because the secondary higher intensity peaks disappeared (Figure 7D). No difference could be observed in the E-cadherin-specific fluorescent signals obtained in both cell lines, treated or not with ET-18-OMe (data not shown).

Figure 7: Flow cytometry of untreated MCF-7/AZ cells (A), MCF-7/AZ cells treated with ET-18-OMe (B), untreated MCF-7/6 cells (C) and MCF-7/6 cells treated with ET-18-OMe (D), labeled with 214D4 monoclonal antibody against episialin followed by rabbit anti-mouse-FITC. Abscissa, relative fluorescence intensity; ordinate, number of cells.
Discussion

ET-18-OMe counteracts loss as well as gain of E-cadherin-dependent functions in two different sublines, MCF-7/AZ and MCF-7/6, of a human breast cancer cell family. It causes gain of E-cadherin function in the adhesion-deficient MCF-7/6 variant as demonstrated by MB2 in the aggregation assays. In the adhesion-proficient MCF-7/AZ variant, ET-18-OMe causes loss of function as evidenced by decreased aggregation and induction of invasion. Episialin is implicated in the loss of E-cadherin function mediated by ET-18-OMe as evidenced by the neutralizing effects of antibodies against episialin. E-cadherin remains, however, the key player as MB2 exerts a dominant effect inducing invasion and reducing adhesion in a way that cannot be neutralized by antibodies against episialin. Functional inactivation of E-cadherin is the mechanism of action of MB2 (Bracke et al., 1993).

How does ET-18-OMe evoke the E-cadherin inhibitory activity of episialin? Since levels of episialin expression were the same in treated as compared to untreated MCF-7/AZ cells, we conclude that ET-18-OMe drives episialin from a neutral (not affecting E-cadherin) to an inhibitory (loss of E-cadherin function) position. Sterical hindrance through its large negatively charged extracellular part is the mechanism by which episialin is reported to inhibit the function of cell surface receptors including E-cadherin and to induce invasion in vivo and in vitro (Ligtenberg et al., 1992; Wesseling et al., 1995; Hilkens et al., 1995b; Wesseling et al., 1996; Mareel and Van Roy, 1998; Kondo et al., 1998). Antibodies cause capping of episialin and so unmask the cell surface receptor.

Changes in the distribution of episialin in ET-18-OMe treated MCF-7/AZ cells are: reduction of the episialin signal in flow cytometry of cells in suspension and co-localisation of E-cadherin with episialin immunosignals in confocal laser scanning of cells on solid substrate. The reduced immunosignal, paralleled neither by immunoblotting, nor by biotinylation, nor by radioimmuno-assay, might be ascribed to a lower accessibility of the antibody, e.g. due to clustering of the antigen bearing the epitope on the non-glycosylated part near the plasmamembrane.

We can only speculate about the mechanism by which ET-18-OMe causes rearrangement of episialin and E-cadherin. Crosslinking of episialin on adjacent cells by the bivalent antibodies is unlikely as the cause of aggregation for the following reasons: In adhesion-proficient MCF-7/AZ cells, in which E-cadherin was neutralized by MB2, neither 214D4 nor M8 antibodies were able to restore adhesion. Furthermore, attempts to crosslink episialin-transfected human melanoma cells by antibody 214D4 failed (Wesseling et al., 1995). Finally, antibodies against episialin did not cause aggregation of adhesion-deficient MCF-7/6 cells. The fact that ET-18-OMe-induced invasion is enhanced by MB2 shows that the invasion-suppressor activity of E-cadherin is not completely neutralized. Interestingly, in the slow aggregation assay, antibodies against episialin restore cell-cell adhesion after ET-18-OMe treatment but not compaction (see Figure 2, panel E). In MCF-7/6 cells, the anti-episialin antibody counteracts ET-18-OMe-induced cell-cell adhesion but not compaction (see smaller but compact aggregates in Figure 2, panel K). These observations point to a role of episialin in E-cadherin-mediated cell-cell adhesion but not or less so in the E-cadherin/catenin mediated signal transduction that results in compaction (Wesseling et al., 1996). Indeed, we found no
evidence for changes in the association between E-cadherin and the catenins. The latter is at variance with the finding that episialin associates with β-catenin in invasive breast cancer (Yamamoto et al., 1997). One possible reason for the rearrangement of transmembrane molecules is increased membrane fluidity ascribed to ET-18-OMe as a consequence of its accumulation in the membrane (van Blitterswijk et al., 1987). Such mechanism was invoked to explain clustering of other transmembrane receptors associated with induction of invasion and loss of tight junction activity (Leroy A., personal communication).

Differences between MCF-7/AZ and MCF-7/6, including the antithetic effects of ET-18-OMe, have been described repeatedly but not explained at the molecular level (Bracke et al., 1991; Bracke et al., 1993; Boterberg et al., 1999b). Our present observations contribute to the understanding of how ET-18-OMe downmodulates E-cadherin functions in the MCF-7/AZ variant. Upregulation of E-cadherin functions in the MCF-7/6 variant, including inhibition of invasion into chick heart fragments, remains to be explained.

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