Role antitumor lipids in cell-cell adhesion and invasion
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CHAPTER 4

Sialylation of E-cadherin does not change the spontaneous or ET-18-OMe-mediated aggregation of human breast cancer MCF-7 cells

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Abstract

We have investigated the role of sialylation on cell-cell adhesion mediated by E-cadherin. Two MCF-7 human breast cancer cell variants were studied: MCF-7/AZ cells showed a spontaneous cell-cell adhesion in the fast and slow aggregation assay, whereas the adhesion deficient MCF-7/6 cell variant failed to form larger aggregates, suggesting that E-cadherin was not functional in the conditions of both assays.

We measured the sialyltransferase activities using Galβ1-3GalNAcα-O-benzyl and Galβ1-4GlcNAcβ-O-benzyl as acceptor substrates as well as mRNA levels of four sialyltransferases, ST3Gal I, ST3Gal III, ST3Gal IV, ST6Gal I, using multiplex RT-PCR in MCF-7 cell variants. The α2-6 and α2-3 sialylation of E-cadherin was investigated by immuno-blot using Sambucus nigra agglutinin and Maackia amurensis agglutinin.

As compared to the adhesion-proficient MCF-7/AZ cells, the adhesion-deficient MCF-7/6 cell line apparently lacks ST6Gal I mRNA, has a lower ST3Gal I mRNA, a lower ST3Gal I sialyltransferase activity, and no α2-3 linked sialic acid moieties on E-cadherin.

The potential anti-cancer drug 1-O-octadecyl-2-O-methylglycero-3-phosphocholine (ET-18-OMe, 48 h, 25 μg/ml) belonging to the class of alkyllysophospholipids restored the E-cadherin function in the adhesion-deficient MCF-7/6 cells as evidenced by an increased aggregation. ET-18-OMe caused loss of ST6Gal I mRNA in MCF-7/AZ cells but no changes of sialyltransferase activities or sialic acid moieties on E-cadherin could be observed.

We conclude that Ca\textsuperscript{2+}-dependent, E-cadherin-specific homotypic adhesion of MCF-7/AZ or MCF-7/6 cells treated with ET-18-OMe was not affected by sialylation of E-cadherin.

Introduction

Invasion, regulated by the dynamic balance between invasion-promotor and invasion-suppressor molecular complexes, marks the difference between benign and malignant tumors [1]. E-cadherin linked by its cytoplasmic part via β-catenin or plakoglobin, and α-catenin to the actin cytoskeleton acts as an invasion-suppressor [2]. The structure and function of the E-cadherin/catenin complex may be disregulated in cancer at various levels, namely by mutations in the cadherin or catenin genes, reduced stability of mRNAs, tyrosine phosphorylation of β-catenin, and by intra- and extracellular associations with other proteins [1, 3-5]. Such disregulation leads to increased invasiveness as documented in experimental as well as clinical cancers [6]. E-cadherin is a transmembrane sialylated glycoprotein [7, 8]. Sialic acids are a family of closely related nine-carbon carboxylated sugars found at the terminal position of mammalian cell surface sugar chains of glycoproteins and glycolipids [9]. Because of their terminal position and their charge, sialylated glycoproteins are important regulators of cellular and molecular interactions [10]. The presence of sialic acid influences the strength of cell-cell adhesion in many systems. In general, the strength of adhesion is weakened by sialic acid [10], and removal of cell surface sialic acid by sialidase increases the adhesiveness between the cells [11-15].
Sialylation of glycoproteins has been implicated in invasion and metastasis although its mechanism of action is not yet completely understood [16, 17]. During development and differentiation of cells, oligosaccharide sequences are strictly controlled [18, 19]. Loss of terminal sequences and sialic acid led to a decrease in metastatic capability in MDAY-D2 mouse lymphoid tumor cells [20]. Li et al. [21] suggested that down regulation of ST6Gal I sialyltransferase in human colon cancer cells by the fecal medium may hamper their invasion and metastasis. In human breast cancer, high expression of sialyltransferases ST3Gal III and ST6Gal I is associated with loss of differentiation and poor prognosis [22]. The role of sialic acid in E-cadherin-mediated cell-cell adhesion has received little attention [7, 8].

1-O-octadecyl-2-O-methylglycero-3-phosphocholine (ET-18-OMe) [23] is an analogue of the naturally occurring 2-lysophosphatidylcholine belonging to the class of alkyllysophospholipids (ALPs) [23]. ALPs interact with cell membranes, thereby interfering with signal transduction mechanisms, modulating the phospholipid metabolism, inducing apoptosis and differentiation and inhibiting invasion [23]. ALPs are known to affect both glycosylation and invasion. ET-18-OMe increased the sialylation of N-glycoproteins in precultured heart fragments (PHF), causing resistance towards invasion of PHF [24]. By contrast, increased sialylation of glycoproteins of rat kidney HSU cells and mouse NIH/3T3 cells endowed these cells with invasive capacities [25].

The effect of ALPs on the E-cadherin-mediated adhesion offered the opportunity to investigate the role of sialic acid in this process. For this purpose we used the MCF-7/AZ variant which is adhesion-proficient in an E-cadherin-dependent manner as well as the MCF-7/6 variant which is adhesion-deficient, as evidenced by aggregation assays [26].

Materials and Methods

Cells

MCF-7/AZ and MCF-7/6 cells are variants of the human mammary carcinoma cell family MCF-7 [26]. 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 cells differ from MCF-7/6 cells in that they are not invasive in vitro when tested in the precultured chick heart invasion assay [26] or the matrigel chemoinvasion assay [27].

Detachment of cells

We used an E-cadherin-saving procedure which is imperative in the preparation of the cells for fast and slow aggregation assay. Cultures were washed three times with Ca²⁺- and Mg²⁺-free Hank’s buffered salt solution (CMF-HBSS) at room temperature; incubated with 3 ml 0.1 U/ml Clostridium hystoliticum collagenase, (Boehringer Mannheim, Mannheim, Germany) 0.04 mM CaCl₂ and 1 g/l glucose in CMF-HBSS at 37°C for 30 min., followed by trypsin/EDTA in modified Puck’s saline (Gibco) at 37°C for 15 min.. Then 1 ml 0.1% soya bean trypsin inhibitor (Sigma, St. Louis, MO) in CMF-HBSS (w/V) with 1 g/l glucose was added.
Antibodies  HECD-1, a mouse monoclonal antibody specific for human E-cadherin, was obtained from Takara Shuzo Co. (Kyoto, Japan). MB2 mouse monoclonal antibody was raised in our laboratory against MCF-7/AZ cells and was found to recognize both the 120 kDa E-cadherin and its 80 kDa trypsin-resistant extracellular part [28].

Assay for cell viability  Cell viability was tested in accordance with Romijn et al. [29]. Briefly, mitochondrial dehydrogenase activities were measured by an MTT-reagent (Sigma). The mean O.D. was reported as a function of increasing ET-18-OMe concentration. In each experiment, 8 cultures were used to determine the mean O.D.. The experiment was repeated at least three times for each cell line.

Assays for cell-cell Aggregation

Slow aggregation assay  This screening assay for cell-cell adhesion was described previously [26]. Briefly, single cell suspensions were seeded onto a semi-solid agar medium. Treatment of cells with MB2 antibody was throughout incubation. After 24 h, aggregate formation was evaluated subjectively under a microscope (Leitz, Wetzlar, Germany) at magnification x 40.

Fast aggregation assay  This is a quantitative assay for cell-cell adhesion as described previously [28]. Briefly, single cell suspensions were incubated under Gyrotrory shaking (New Brunswick Scientific, New Brunswick, NJ) at 80 rpm for 30 min. in an isotonic buffer containing 1.25 mM Ca\(^2^+\). Treatment of cells at 4°C with MB2 antibody was started 30 min before the aggregation period and continued throughout aggregation at 37°C. The volume % distribution in function of the particle diameter was measured by a 200 LS Coulter particle size counter (Analis, Namen, Belgium) at the start of the incubation (N0) and after 30 min of the incubation (N30).

Multiplex R T-PCR of four sialytransferases  A multiplex RT-PCR has been developed to monitor the expression of four sialyltransferases [30]. Total RNA of MCF-7/AZ and MCF-7/6 cells, treated or not with ET-18-OMe was extracted using the guanidium thiocyanate method described by Sambrook et al. [31] and cellular RNA was quantified by spectrophotometry at 260 nm. Briefly, five μg of total cellular RNA was used for reverse transcription into cDNA. Two μl of the reverse transcription reaction were subjected to PCR amplification using sialyltransferases or GAPDH specific primers. The 23 μl PCR mixture consisted of 1 U Hi-Taq DNA polymerase, 10 mM Tris-HCl pH 8.8, 1.5 mM MgCl\(_2\), 50 mM KCI, 0.1% Triton X-100, 0.2 mM dNTP, and 0.6 μM of each primer. Samples were overlaid with two drops of mineral oil (Sigma). Reactions were run in a PTC-100™ thermal cycler (MJ Research, Watertown, USA) using the following conditions: 1 min at 94°C, 1 min. 58°C, 1.5 min 72°C for 36 cycles. Ten μl aliquots of the PCR reaction were size-separated on a 10% polyacrylamide gel equilibrated in Tris Base 0.13 M, boric acid 45 mM (TBE). Gels were stained with ethidium bromide (1 μg/ml), photographed using Polaroid film under UV light. Size of the generated fragments were estimated according to the migration of a 1kb DNA ladder. The experiment was repeated at least three times.
**Sialyltransferase assays**

**Preparation of cell homogenates** Cells from confluent cultures were detached as described above; they were washed three times with phosphate buffered saline (PBS) and lysed with a solution of 0.25 M sucrose and 0.2 M NaCl. After 5 min centrifugation at 3,000 x g, the pellets were discarded and the supernatants were centrifuged at 105,000 x g for 1 h. The microsomal fractions were solubilized in 500 µl of 10 mM sodium cacodylate, pH 6.5, containing 1% Triton X-100, 20% glycerol, 5 mM dithiothreitol and 5 mM MnCl$_2$ and used for enzyme assays.

**Galβ1-3GalNAcα-O-benzyl and Galβ1-4GlcNAcβ-O-benzyl as substrates** The substrate Galβ1-3GalNAcα-O-benzyl (Sigma) was used to determine the sialyltransferase activity of CMP-sialic acid: Galβ1-3GalNAcα2,3-sialyltransferase (ST3Gal 1; EC 2.4.99.4) which is responsible for α2-3 sialylation of O-linked core 1 glycoproteins and the substrate Galβ1-4GlcNAcβ-O-benzyl was used to determine the sialyltransferase activity of CMP-sialic acid: Galβ1-4GlcNAcα2,6-sialyltransferase (ST6Gal 1; EC 2.4.99.1) which is responsible for α2-6 sialylation of N-linked glycoproteins [9]. For these enzyme activities, aliquots of the microsomal suspension (50 µl, corresponding to 21 - 72.5 µg of protein) were brought to a final volume of 180 µl, with 0.1 M sodium cacodylate pH 6.5, 0.8 % Triton X-100, 0.2 M galactose (to inhibit the β-galactosidases), 1 mM of 2,3-dehydro-2-deoxy-Neu5Ac (to inhibit the sialidases), 53 µM CMP-[14C]-Neu5Ac (0.58 Gbq/mmol; 5.5 kBq/180 µl) (Sigma), containing 1 mM of Galβ1-3GalNAcα-O-benzyl or Galβ1-4GlcNAcβ-O-benzyl. The reactions were carried out in duplicate for 1 h and the reaction was stopped by adding 1 volume of ethanol, followed by centrifugation at 4,000 rpm, 4°C for 4 minutes and the supernatant was then purified on 1 ml C$_8$ columns Waters Corp., Milford, MA). Therefore, the columns were activated with 5-10 ml methanol and washed with 10-20 ml water. After loading the supernatant on the column, followed by washing with 10 ml water, the [14C]NeuAcα2-3Galβ1-3GalNAcα-O-benzyl or [14C]NeuAcα2-6Galβ1-4GlcNAcβ-O-benzyl was eluted with 10 ml acetonitril 30% and prepared for scintillation counting in a LS3801 scintillation counter (Beckman, CA).

**Sialylation of glycoproteins** SDS/PAGE was performed on a 4-20% gradient gel under reducing conditions using 20 µg protein of total cell lysates. Western-blotting on nitrocellulose membranes Biotrace NT (Gelman Science, Ann Arbor, MI), was performed using standard procedures [32]. Each blot was then treated with polyvinylpyrrolidone (2% in TBS) prior to the incubation in TBS containing 1 mM MgCl$_2$, 1 mM MnCl$_2$, 1 mM CaCl$_2$ with the digoxigenin-labeled lectins : SNA-dig (2µg/ml), and MAA-dig (5 µg/ml) (Boehringer Mannheim). Incubations were performed with and without treatment of 50 mU/ml of sialidase from *Clostridium perfringens* (Sigma) (in 50 mM citrate buffer pH 6.0, 0.9 % NaCl, 0.1 % CaCl$_2$) for 16 h at 37°C. After washing, blots where incubated for 1h with anti-digoxigenin alkaline phosphatase-labeled Fab fragments (Boehringer Mannheim) (1 µg/ml TBS) and labeled glycoproteins were revealed by NBT/X-phosphate staining according to Boehringer Glycan Detection Kit protocol with minor modifications [33].
**Immunoprecipitation of E-cadherin**

Cells from confluent cultures were detached as described above. They then were washed three times with PBS and lysed with 1 ml of PBS containing 1% triton X-100, 1% NP-40, 0.9 mM Ca\(^{2+}\), 0.33 mM Mg\(^{2+}\), 300 µg/ml PMSF, 10 µg/ml leupeptin and 10 µg/ml aprotinin. 250 µl of lysate was cleared by the addition of 100 µl of a concentrated suspension of protein G-sepharose beads (Pharmacia, Uppsala, Sweden), and subsequent rotary shaking at 4°C during 30 min. 1 µg HECD-1 was added to the supernatant, and the whole was incubated at 4°C for 3 h. Then 50 µl of a protein G-sepharose bead suspension was added and the incubation prolonged for 1 h. The beads were washed three times with PBS, containing 0.1% triton X-100, 0.1% NP-40, and the protein was eluted with Laemmli buffer [34]. The eluate was subjected to immunoblot analysis of E-cadherin using SDS/PAGE on 4-20% gradient gels under reducing conditions. HECD-1 antibody (dilution 1/10) was used to reveal the E-cadherin. Sialic acid on E-cadherin was revealed as mentioned in ‘Sialylation of glycoproteins’.

**Statistics**

The number of cultures used in the experiments is indicated in the Results section. The student t-test was used for the comparison of sialyltransferase activities in MCF-7/AZ and MCF-7/6, treated or not with ET-18-OMe.

**Results**

**Effect of ET-18-OMe on cell viability**

MCF-7/AZ and MCF-7/6 cells showed a similar dose-dependent decrease in viability after a 48 h treatment with ET-18-OMe as evidenced by the MTT test (Figure 1). For all further experiments we chose a concentration of 25 µg ET-18-OMe/ml, leaving 80% of the cells viable.

![Graph showing MTT test results](image)

**Figure 1:** MTT-test of MCF-7/AZ (closed squares) and MCF-7/6 (open squares) cells, after treatment with ET-18-OMe for 48 h is shown. Ordinate: Optical Density (O.D.) ; mean values from 16 cultures, normalized to 100% for untreated cultures. Abscis, concentration of ET-18-OMe (µg/ml).
Aggregation of MCF-7 cells

Figure 2: Aggregation of MCF-7/AZ and MCF-7/6 on semi-solid agar after (A) MCF-7/AZ, (B) MCF-7/AZ treated with 25 μg/ml ET-18-OMe, (C) MCF-7/AZ + MB2, (D) MCF-7/AZ treated with 25 μg/ml ET-18-OMe + MB2, (E) MCF-7/6, (F) MCF-7/6 treated with 25 μg/ml ET-18-OMe, (G) MCF-7/6 + MB2, (H) MCF-7/6 treated with 25 μg/ml ET-18-OMe + MB2. Scale bar = 0.5 mm.
The slow aggregation assay revealed a low degree of cell-cell adhesion in MCF-7/6 cells in contrast with MCF-7/AZ cells. After 24 h culture, the former produced numerous smaller and more irregular aggregates (Figure 2E) whereas the latter formed larger and more coalescent aggregates (Figure 2A). Treatment with ET-18-OMe led to a decrease in MCF-7/AZ cell-cell adhesion with the formation of small and irregular aggregates (Figure 2B) and to an increase in MCF-7/6 cell-cell adhesion with the formation of large aggregates (Figure 2F). Addition of MB2 antibody, functionally blocking E-cadherin [19], invariably resulted in small and irregular aggregates (Figures 2C, 2D, 2G, 2H).

The fast aggregation assay (Figure 3) confirmed the data obtained with the slow aggregation assay.

![Differential Volume](image)

Figure 3: Cell-cell adhesion of MCF-7/AZ and MCF-7/6 cells, treated or not with ET-18-OMe. Cell suspensions prepared under E-cadherin-saving conditions were allowed to aggregate in Ca\(^{2+}\)-containing medium. The particle diameter was measured after 30 min. incubation for the MCF-7/AZ cells (Fig. 3A) with, (1) MCF-7/AZ, (2) MCF-7/AZ treated with ET-18-OMe, (3) MCF-7/AZ with MB2 (monoclonal antibody against E-cadherin) and for the MCF-7/6 cells (Fig. 3B) with (1) MCF-7/6, (2) MCF-7/6 treated with ET-18-OMe, (3) MCF-7/6 treated with ET-18-OMe and MB2.
Both the spontaneous MCF-7/AZ (Figure 3 - ①) and the ET-18-OMe-mediated MCF-7/6 cell-cell adhesion (Figure 3B - ②) were inhibited by MB2 (Figure 3A - ②, Figure 3B - ③). In MCF-7/AZ cells, ET-18-OMe mimicked the effect of the anti-E-cadherin antibody MB2 (Figure 3A - ③). These results suggest that ET-18-OMe activates E-cadherin-dependent intercellular adhesion of MCF-7/6 cells.

**The RT-PCR of four sialyltransferases** The expression pattern of the four sialyltransferases ST3Gal I, ST3Gal IV, ST6Gal I and ST3Gal III was analyzed in total RNA by multiplex in three independent experiments. A typical and highly reproducible profile of the signals obtained after loading the cDNAs on a polyacrylamide gel is shown in Figure 4.

![Figure 4](image)

**Figure 4**: RT-PCR co-amplification of the four sialyltransferases cDNAs: ST3Gal I, ST3Gal IV, ST6Gal I, ST3Gal III, from the breast cancer cells RNA: MCF-7/AZ (lane 1), MCF-7/AZ + ET-18-OMe (lane 2), MCF-7/6 (lane 3), MCF-7/6 + ET-18-OMe, (lane 4). The products of 36 cycles of amplification performed according to Materials and Methods were run at 10% polyacrylamide gel and ethidium-bromide-stained. The localisation of specific fragments for ST3Gal I (537 bp), ST3Gal IV (458 bp), ST6Gal I (371 bp), ST3Gal III (300 bp) are indicated at the right side of the figure. The products of amplification of the unregulated gene GAPDH are shown on the bottom of the figure.
The expression patterns of the sialyltransferases ST3Gal IV and ST3Gal III were similar for MCF-7/AZ and MCF-7/6 (Figure 4) and no changes were obvious after treatment with ET-18-OMe. The expression of ST3Gal I, responsible for α2-3 sialylation of Galβ1-3GalNAc, was lower in MCF-7/6 than in MCF-7/AZ cells. ET-18-OMe had no influence on expression of ST3Gal I. Expression of the ST6Gal I sialyltransferase was demonstrated in MCF-7/AZ cells but not in MCF-7/6 cells. The ST6Gal I band was no longer visible when MCF-7/AZ cells were treated with ET-18-OMe.

Sialyltransferase assay  The sialyltransferase activities were assayed, using as substrates Galβ1-3GalNAcβ-O-benzyl and Galβ1-4GlcNAcα-O-benzyl (Table 1).

Table 1: Sialyltransferase activity of MCF-7/AZ and MCF-7/6 homogenates, treated or not with ET-18-OMe. Incubations were performed as described in Materials and Methods. Galβ1-3GalNAcα-O-benzyl and Galβ1-4GlcNAcβ-O-benzyl were used as acceptor substrates. Results are expressed as mean values in pmol of [14C]-NeuAc residues transferred per µg of protein per hour.

<table>
<thead>
<tr>
<th>Acceptor substrate</th>
<th>MCF-7/AZ</th>
<th>MCF-7/AZ + ET-18-OMe</th>
<th>MCF-7/6</th>
<th>MCF-7/6 + ET-18-OMe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galβ1-3GalNAcα-O-benzyl</td>
<td>5.70 ± 0.05</td>
<td>3.29 ± 0.10</td>
<td>1.10 ± 0.06</td>
<td>1.51 ± 0.04</td>
</tr>
<tr>
<td>Galβ1-4GlcNAcβ-O-benzyl</td>
<td>0.08 ± 0.03</td>
<td>0.09 ± 0.02</td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.01</td>
</tr>
</tbody>
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ST3Gal I activity was significantly higher in MCF-7/AZ than in MCF-7/6 cells (statistically significant, T-test, p<0.05); the enzymatic activity of ST6Gal I was very low in both cell lines. Treatment of the cells with ET-18-OMe did not influence these enzymatic activities in a statistically significant manner.

Sialylation of glycoproteins  20 µg proteins from total cell lysates were separated by SDS/PAGE, electroblotted and probed with lectins (Figure 5).
Figure 5: *Sambucus nigra* agglutinin (SNA) (A) and *Maackia amurensis* agglutinin (MAA) (C) were used to detect α2-6 and α2-3 linked sialic acid respectively, on total cell lysates of MCF-7/AZ and MCF-7/6 cells, separated via SDS-PAGE (4-20%) and electroblotted on nitrocellulose. As a control for aspecific binding of the lectins, a sialidase treatment was carried out followed by staining with SNA (B) and MAA (D).

In all cases, α2-6 sialylation of N-and O-linked glycoproteins by SNA and α2-3 sialylation of N-and O-linked glycoproteins by MAA was observed. The α2-6 sialylation was weak for both cell types and no significant changes were observed after treatment with ET-18-OMe (Figure 5A). In MCF-7/AZ cells, ET-18-OMe caused an increase in binding of MAA (Figure 5C). In MCF-7/6 cells treatment with ET-18-OMe (Figure 5C) had no effect on sialylation signals. No aspecific binding of the lectins SNA (Figure 5B) and MAA (Figure 5D) occurred, as evidenced by treatment with sialidase.
Sialylation of E-cadherin  Immunoprecipitation and immunoblotting of ET-18-OMe treated or untreated MCF-7/AZ and MCF-7/6 cell extracts with the monoclonal antibody HECD-1 revealed an intact E-cadherin band at 120-kDa (Figure 6A).

![Image of immunoblot analysis](image_url)

**Figure 6**: Immunoblot analysis of E-cadherin in MCF-7/AZ and MCF-7/6 cells treated or not with ET-18-OMe. Cell extracts were separated via SDS-PAGE (4-20%) and electroblotted onto nitrocellulose membranes. E-cadherin was revealed as a 120 kDa (A) band by monoclonal antibody HECD-1. *Sambucus nigra* agglutinin (SNA) (B) and *Maackia amurensis* agglutinin (MAA) (C) were used to reveal α2-6 and α2-3 linked sialic acid respectively, on E-cadherin.

The lectins SNA and MAA revealed a band of α2-6 linked sialic acid (Figure 6B) and α2-3 linked sialic acid (Figure 6C) respectively, which coincided with the HECD-1-positive E-cadherin band. In MCF-7/AZ cells, E-cadherin is mainly α2-3 sialylated as revealed by MAA, while in MCF-7/6 cells SNA revealed mainly α2-6 sialylation. Neither in MCF-7/AZ nor in MCF-7/6 cells, we observed an effect of ET-18-OMe on sialylation of E-cadherin.
Discussion

The MCF-7/AZ cells showed a spontaneous cell-cell adhesion, whereas the adhesion deficient MCF-7/6 variant failed to form larger aggregates. Both cell lines reacted differently to treatment with ET-18-OMe. The MCF-7/AZ cells formed smaller aggregates while the MCF-7/6 cells formed larger aggregates.

Similar dual effects on in vitro behaviour were obtained with other agents as well as with ET-18-OMe. Pretreatment with pertussis toxin (Ptx) of the highly metastatic and invasive mouse T-lymphoma BW-O-Li cells inhibited invasion, whereas the same pretreatment of the non-metastatic non-invasive parental BW 5147 cells rendered them invasive, as measured by the monolayer invasion assay [35]. Retinoic acid (RA) inhibited both ruffling and invasion of MCF-7/6 cells whereas the opposite effect was found with the non-invasive MCF-7/AZ cells. Since the number of plasmin receptors was higher on the invasive MCF-7/6 cells as compared with the non-invasive MCF-7/AZ cells, the authors suggested a possible correlation between the effects of RA on invasion and on proteolysis of the extracellular matrix [19]. 12-O-tetradecanoylphorbol 13-acetate (TPA) inhibited the invasiveness of malignant human fibrosarcoma HT 1080 in Matrigel, whereas the non-invasive lung fibroblast WI-38 cells were stimulated to invade. The effects of TPA were correlated with cell motility as well as with collagenase IV activity [36]. ET-18-OMe inhibited the invasion of malignant mouse fibrosarcoma MO4 cells in organ culture [37]. Pretreatment with ET-18-OMe of the non-invasive Madin Darby Canine Kidney MDCK cells render them invasive [38], pretreatment of the chick heart host tissue blocked the invasion of the confronting malignant cells [25]. All of these changes on invasion were correlated with an altered glycosylation such as increased branching and/or degree of sialylation [25, 37, 38].

E-cadherin has been implicated in the differential behaviour of both MCF-7/AZ and MCF-7/6 cells. Levels of E-cadherin are not affected by ET-18-OMe. Nevertheless, results with the antibody MB2 suggest that ET-18-OMe activates E-cadherin-dependent adhesion of MCF-7/6 cells as it operates in MCF-7/AZ cells. Whether or not ET-18-OMe-mediated decrease in aggregation implicates E-cadherin is hard to demonstrate.

The aim of our experiment was to examine the role of sialylation in the E-cadherin-mediated changes in the aggregation of the MCF-7 variants. Our results show that differences in overall sialylation exist between the two variants. MCF-7/AZ cells have higher ST3Gal I and ST6Gal I mRNA levels and a higher ST3Gal I enzymatic activity than MCF-7/6 cells. This ST6Gal I mRNA signal in MCF-7/AZ cells disappeared after treatment with ET-18-OMe. It is hard to accept that differences in ST6Gal I are responsible for the differential behaviour of the MCF-7 variants, since ET-18-OMe clearly changed MCF-7/6 cells adhesive phenotype without changing the ST6 Gal I.

Compared to the MCF-7/6 variant, treatment with ET-18-OMe of MCF-7/AZ cells caused an increased MAA-binding, while ET-18-OMe induced changes could not be observed for ST3Gal I mRNA or ST3Gal I sialyltransferase. We suggest that the increase in MAA-binding is not due to an ‘hypersialylation’ but to an increase in the acceptor sites for α2-3 sialylation (unpublished results). Bolscher et al. described this effect with ET-18-OMe in rat and mouse cells and
suggested that it was due to more branching of the glycoproteins rather than changes in sialylation [25]. These alterations can be responsible for the decrease in cell-cell adhesion [25, 39].

As to the investigation of sialylation, it is difficult to remove the sialic acid moieties from e.g. E-cadherin without changing the sialylation of other molecules. In our assays, aggregation (gain of function) was shown to be E-cadherin-dependent through the use of functionally inactivating antibodies. However, it is more difficult to demonstrate the specificity of failure of aggregation (loss of function).

Shore et al. [7] suggested that E-cadherin is mainly N-glycosylated but that this glycosylation is not necessary for the surface expression of the protein. Deman et al. [8] observed α2-6 sialic acid moieties on E-cadherin of MCF-7/6 cells. After removal of sialic acid with sialidase from MCF-7/AZ cells, or from MCF-7/6 cells induced to aggregate by IGF-I on the fast aggregation assay, showed reduced cell-cell adhesion whereas the slow aggregation assay showed no changes. The authors [8] suggested that sialic acid is needed for theinstalment of the weak intercellular bonds that initiate aggregation. Whether the loss of cell-cell adhesion was due to the removal of sialic acid from E-cadherin or from other glycoproteins was not clear.

We observed the presence of α2-6 sialic acid on E-cadherin of MCF-7/6 cells, in accordance with Deman et al. [8] and α2-3 sialic acid on E-cadherin of MCF-7/AZ cells. This was, however, not changed by treatment with ET-18-OMe that clearly altered cell-cell adhesion. The levels of ST3Gal I mRNA fitted well with the sialyltransferase activity typical of ST3Gal I measured using Galβ1-3GalNAca-O-benzyl as acceptor substrate and also with an α2-3 sialylolation of total glycoproteins including E-cadherin.

Levels of ST6Gal I mRNA were more or less undetectable. A signal was found in RT-PCR products from MCF-7/AZ but not from MCF-7/6 cells and this weak signal disappeared with treatment of ET-18-OMe. In parallel, sialyltransferase activity relevant for ST6Gal I was very low expressed (<0.1 pmol/μg protein.h) in these cells but SNA binding visualized an α2-6 sialylation of glycoproteins in both cell lines. This apparent discrepancy could be explained by the fact that using SNA we revealed sialic acid α2-6 linked to Gal but also to GalNAc, the latter occurring in O-glycans, while ST6Gal specificity is restricted to Galβ1-4GalNAc disaccharide sequence. The sialyltransferases responsible for α2-6 sialylation of O-linked GalNAc (ST6GalNAc) have not yet been cloned from human [9] and cannot therefore be investigated by RT-PCR. The SNA binding could nevertheless reflect the presence of NeuAcα2-6GalNAc sequence (sialyl Tn antigen) which is known to be expressed in breast cancer on both MCF-7/AZ and MCF-7/6 glycoproteins [40].

We suggest that the dual effect on adhesion is due to functional inhibition (MCF-7/AZ) or stimulation (MCF-7/6) of E-cadherin and that sialylation of E-cadherin does not play a crucial role in the functionality of E-cadherin for both MCF-7/AZ or MCF-7/6 cells.
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