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REMOVAL OF SIALIC ACID FROM THE SURFACE OF HUMAN MCF-7 MAMMARY CANCER CELLS ABOLISHES E-CADHERIN-DEPENDENT CELL-CELL ADHESION IN AN AGGREGATION ASSAY

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SUMMARY

MCF-7 human breast cancer cells express E-cadherin and show, at least in some circumstances, E-cadherin-dependent cell-cell adhesion (Bracke et al., 1993).The MCF-7/AZ variant spontaneously displays E-cadherin-dependent fast aggregation; in the MCF-7/6 variant, E-cadherin appeared not to be spontaneously functional in the conditions of the fast aggregation assay, but function could be induced by incubation of the suspended cells in the presence of insulinlike growth factor I (IGF-I) (Bracke et al., 1993).

E-cadherin from MCF-7 cells was shown to contain sialic acid. Treatment with neuraminidase was shown to remove this sialic acid, as well as most of the sialic acid present at the cell surface. Applied to MCF-7/AZ, and MCF-7/6 cells, pretreatment with neuraminidase abolished spontaneous as well as IGF-I induced, E-cadherin-dependent fast cell-cell adhesion of cells in suspension, as measured in the fast aggregation assay. Treatment with neuraminidase did not, however, inhibit the possibly different, but equally E-cadherin-mediated, process of cell-cell adhesion of MCF-7 cells on a flat plastic substrate as assessed by determining the percentage of cells remaining isolated (without contact with other cells) 24 h after plating.

Key words: sialic acid; E-cadherin; cell-cell adhesion; IGF-I; MCF-7 cells.

INTRODUCTION

Sialic acid (N-acetylneuraminic acid) is a negatively charged carbohydrate that is found in the oligosaccharide chains of many glycoproteins and glycolipids on the cell surface. The presence of sialic acid has been found to influence the strength of cell-cell adhesion in many adhesion systems. In the majority of these, the strength of adhesion (Bell et al., 1984) is weakened by sialic acid, and removal of cell surface sialic acid by neuraminidase increases the adhesiveness between the cells. This fact has been demonstrated in instances where the actual adhesion molecules were unknown (Deman et al., 1974; Ligtenberg et al., 1992) or had been characterized (Rutishauser et al., 1988; Springer, 1990; Acheson et al., 1991).

An exception thus far are the heterotypic cell-cell adhesions in which adhesion molecules of the selectin superfamily are involved. Here the presence of cell surface sialic acid is needed for the formation of the adhesive bonds, and treatment with neuraminidase abolishes cell-cell adhesion (Lasky, 1992).

In cell-cell adhesion systems where the homophilic cadherins are the dominant adhesion molecules (Takeichi, 1988), a role for sialic acid has not yet been described. Here we report on E-cadherin-mediated aggregation in two MCF-7 human breast cancer cell variants. In one of these variants, the cells showed spontaneous cell-cell adhesion in a fast aggregation assay, whereas in the other variant, fast aggregation could be induced by insulinlike growth factor I. Our aim was to investigate whether cell surface sialic acids were involved in the establishment of intercellular adhesive bonds in these two cell lines.

MATERIALS AND METHODS

For a more detailed description of some procedures and materials we refer to earlier papers (Bracke et al., 1991, 1993).

Cells. MCF-7/6 and MCF-7/AZ are variants of the MCF-7 human mammary carcinoma cell family. The cells are maintained on tissue culture plastic substrate in different media. The one for MCF-7/6 is a mixture of Dulbecco's modified Eagles medium (DMEM) and HAM F12 (50/50) supplemented with 0.05% (wt/vol) glutamine, 250 IU ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin, and 10% fetal bovine serum (FBS). The medium for MCF-7/AZ is Eagle's minimum essential medium (EMEM) with the same amounts of glutamine, penicillin, and streptomycin as above, and 5% FBS with the additional supplementation of 6 ng ml^{-1} bovine insulin. 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 fragment invasion assay (Bracke et al., 1991) and the matrigel chemoinvasion assay (Simon et al., 1992).

Antibodies. HECD-1, a mouse monoclonal antibody specific against human E-cadherin, was a gift from Dr. Y. Shimoyama (National Cancer Research Institute, Tokyo, Japan) or was obtained from Takara Shuzo Co. (Kyoto, Japan). Depending on the type of experiment, different dilutions were made from the original supernatants. MB2 mouse monoclonal antibody was raised in our laboratory against MCF-7/AZ cells and was found to recognize both the 120-kD E-cadherin- and its 80-kD trypsin-resistant extracellular part (Bracke et al., 1993). 5D10 mouse monoclonal antibody against MCF-7 cells (Plessers et al., 1986), was a gift from Dr. L. Plessers (Limburgs Universitair Centrum, Diepenbeek, Belgium). The original supernatants of the MB2 and 5D10 producing cells were diluted 1/20 in the present experiments. Prior to

the measurement of their aggregation rate, cells were incubated with antibodies at 4° C for 30 min. Subsequent cell handling was at 37° C in the presence of antibody.

Other chemicals. Human recombinant insulinlike growth factor I (IGF-I) was from Boehringer Mannheim Biochemicals (Mannheim, Germany). Neuraminidase (from Vibrio Cholerae, 5 U/ml) was from Serva (Heidelberg, Germany).

Immunodetection of E-cadherin. Immunocytochemical localization of E-cadherin was done on glass coverslips after fixation with methanol at 20° C. The primary monoclonal antibodies were revealed with secondary rabbit anti-mouse FITC (RAM-FITC) antiserum (Dako Corporation, Glostrup, Denmark). Indirect immunofluorescence coupled to flow cytometry was performed on 5 $\times 10^5$ cells after detachment from plastic substrate using the E-cadherin-saving procedure (*see* below). The cells were incubated with HECD-1 (dilution 1/20) at 4° C for 1 h. Then a RAM antiserum conjugated with fluoresceine isothiocyanate (FITC: Dako) was added. The fluoresceine intensity was measured with a FACScan III (Becton Dickinson, Mountainview, CA). Immunoblot analysis of E-cadherin in cell extracts or in immunoprecipitates was made with polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE; 7.5% wt/vol). HECD-1 antibody (dilution 1/10) was used to reveal the E-cadherin. Sialic acid was revealed with the Sambucus niger bark lectin (SNA, digoxigenin labeled; 1 μg/ml) staining method (Boehringer, Mannheim). Electroblotting was done on Immobilon membranes (Millipore Corp., Bedford, MA).

Detachment of cells. In some experiments, the cells were detached from their tissue culture plastic substrate by a trypsin/EDTA solution (0.25% trypsin, 0.02% EDTA, 0.9% NaCl). This procedure, which is destructive for cell surface glycoproteins, yields suspended cells with low mutual adhesiveness, and they can be easily reseeded as new cultures on a plastic substrate. In the other instances, we used an E-cadherin-saving procedure, which has been described in detail (Bracke et al., 1993; Mareel et al., 1994). The method is based on treatment of the cells in 75cm² plastic culture vessels with collagenase followed by treatment with trypsin (0.01%, wt/vol) in the presence of a low calcium concentration (0.04 m*M*). Besides E-cadherin, other molecules are retained intact also, among which the IGF-I receptor. The use of this method is imperative in the preparation of the cells for the fast aggregation assay.

Immunoprecipitation of E-cadherin. Cells from confluent cultures were detached as described above. They then were washed three times with phosphate buffered saline (PBS) and lysed with 1 ml of PBS containing 1% Triton X-100, 1% NP-40, 0.9 m*M* Ca^{2+}, 0.33 m*M* 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 rotatory 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 and 0.1% NP-40, and the protein was eluted with Laemmli buffer. The eluate was subjected to immunoblot analysis as described above.

Cell-cell adhesion. a) Assay for cell scattering on a flat substrate: Cells were detached from their plastic substrate by trypsin/EDTA. 2 ml of suspension containing 160 000 cells in their culture medium supplemented with 1% FBS was brought into 6-well plastic plates (Nunc, Roskilde, Denmark). After the addition of MB2 antibody or of supernatant of a nonproducing hybridoma culture (dilution 1/20), the cells were incubated for 24 h at 37° C, and then real time video-recordings of 15 or more microscopic fields per condition were made through an inverted microscope. For each field, the percentage number of isolated cells was determined. b) Fast aggregation assay. For the measurement of the degree of aggregation between cells in suspension, cells that had been detached from their plastic substrate by the E-cadherin saving procedure, were eventually incubated with neuraminidase (*see* further) and/or IGF-I, and were then subjected to gyratory shaking. The IGF-I concentration was 0.5 μg/ml and incubation took place for 30 min at 37° C. Further processing then was in the presence of IGF-I. The number of particles after 30 min of shaking (N_{30}) was counted in a Coulter counter (Coulter, Harpenden, England) using an orifice of 400 μm, and divided by the particle count at the start of the gyratory shaking (N_0) (Bracke et al., 1993). The cell viability just before the measurement of the aggregation rate had not declined to an appreciable extent.

Prior to neuraminidase treatment, detached cells were centrifuged and resuspended in growth medium without phenol red but supplemented with 1% FBS. To a 2-ml sample containing 10^7 cells, 1 unit of neuraminidase in acetate buffer pH 5.5 was added, and the suspension was incubated for 30 min at 37° C with occasional shaking. Cells that did not receive neuraminidase treatment were incubated in the same conditions except for the presence of the enzyme. After the cells had been treated with neuraminidase, we also added the enzyme (0.1 unit ml^{-1} to the Hanks' buffered salt solution (HBSS) in which the cells were shaken in preparation for the measurement of the aggregation rate.

A non-enzymatic removal of sialic acid from the cell surface was performed by treating the cells with 0.05 *M* H_2SO_4 at 80° C during 1 h.

For the determination of sialic acid, the cells were treated with neuraminidase as described above. The amount of sialic acid was determined on the supernatant after centrifugation of the cells. The method of Warren (1959) was followed with a correction for the presence of glycoproteins (Deman et al., 1973).

For protein determination, the method of Bradford (1976) was followed. The viability of the cells was determined qualitatively with the explant test: 1 drop of the cell suspension was added to 2 ml of growth medium in a 18-mm well (Nunc). Spread cells after 2 h and 24 h of incubation at 37° C were considered to be viable.

Results

Expression of E-Cadherin and Its Role in Cell-Cell Adhesion

Both MCF-7/AZ and MCF-7/6 cells were found to express E-cadherin as shown by: a) Immunocytochemical staining with the anti-E-cadherin antibodies MB2 and HECD-1; cells grown on tissue culture plastic substrate or on glass form epitheloid islands showing a distinct immunoreactivity at the site of cell-cell contacts, confirming previous observations respectively by Bracke et al. (1993) and Mareel et al. (1994). b) Western blotting of total cell extracts in which E-cadherin was revealed by the use of HECD-1 antibody; c) Indirect immunofluorescence coupled to flow cytometry allowing for quantitation of bound HECD-1 antibody.

That E-cadherin is involved in cell-cell adhesion between MCF-7 cells was shown using both the assay for cell scattering on a flat substrate and the fast aggregation assay. Results with the assay for cell scattering: seeding the MCF7/AZ or MCF-7/6 cells in medium that contained MB2 antibody against E-cadherin yielded cultures in which most cells remained solitary after 24 h (Table 1 *A*, Fig. 1 *A*); the cells were scattered over the substrate with few intercellular contacts; in cultures that contained no antibody, few solitary cells were found; cells in cultures that contained MB2 antibody were still viable 4 d after seeding. Results with the fast aggregation assay: in line with the functional dependency of cadherins on calcium, MCF-7/AZ cells were found to aggregate in the presence of 1.25 m*M* of calcium but not in the absence of the cation (Fig. 2 *A*); treatment with HECD-1, a specific antibody against E-cadherin, results in absence of aggregation; adding IGF-I when incubating in the presence of 1.25 m*M* of calcium did not affect the result; MCF-7/6 cells failed to aggregate spontaneously in the presence of calcium ions, but, as previously demonstrated (Bracke et al., 1993) aggregation between suspended MCF-7/6 cells could be induced by IGF-I, and this induced aggregation could be inhibited by pretreating the cells with the anti-cadherin antibody HECD-1 (Fig. 2 *C*); incubation of the cells with IGF-I in the absence of calcium ions yielded nonaggregating cells when measured in the presence of calcium ions (not shown).

Sialic Acid, E-Cadherin, and Cell-Cell Adhesion

E-cadherin contains sialic acid. Using Western blotting and a sialic acid-specific lectin, we showed that E-cadherin from MCF-7/

TABLE 1

EFFECT OF MB2 ANTIBODY AND NEURAMINIDASE ON CELL SCATTERING ON A PLASTIC SUBSTRATE[a]

	SOLITARY CELLS AS PERCENTAGE OF TOTAL CELLS	
A	MB2 antibody	control
MCF-7/AZ	85.7 (± 8.2)*	14.1 (± 10.7)
MCF-7/6	91.3 (± 11.7)	5.2 (± 5.5)
B	neuraminidase	control
MCF-7/AZ	5.6 (± 8.6)	3.1 (± 5.1)
MCF-7/6	20.9 (± 17.2)	17.9 (± 12.8)

[a]The cells were detached from tissue culture plastic substrate with a trypsin/EDTA solution and *A*, seeded on a plastic substrate in a medium that contained MB2 antibody against E-cadherin. In the controls, the medium was supplemented with supernatant from non-MB2 producing cells. As another control, we substituted the MB2 antibody in an MCF-7/AZ culture against the general 5D10 antibody that recognizes MCF-7/6 cells. The percentage of solitary cells in that case was 17.5 (± 19.3). *B*, The cells were pre-incubated for 30 min at 37°C with neuraminidase (1 U/10^7 cells), and then seeded. The growth medium contained 1% FBS and a seven times lower neuraminidase concentration as that used during pretreatment. The control cells were pre-incubated and cultured in absence of neuraminidase.

* values are means ± s.d. of 15 to 20 separate observations. The p-values of the experiments under *A* were all smaller than 0.001. The experiments under *B* had p-values of 0.1014 (MCF-7/AZ) and 0.7354 (MCF-7/6)

6 cells contains sialic acid. Western blotting (Fig. 3) of an extract of MCF-7/6 cells revealed E-cadherin as a 120-kD electrophoretic band. The position of the band (*lane 1*) is compatible with the molecular weight of intact human E-cadherin. E-cadherin in the cell extract was immunoprecipitated with HECD-1. The precipitate was resolubilized with detergent and then applied on a strip for electrophoresis. Sialic acid on the electroblot was revealed by the specific SNA reagent (*lane 2*). Its location coincided with the E-cadherin band in the Western blot of the whole cell. We also performed immunoprecipitation with HECD-1 on E-cadherin from extracts of cells that had been treated with neuraminidase. In that case (*lane 3*), SNA staining at the location of the E-Cadherin band was absent. The 59-kD bands in *lanes 2* and *3* can be ascribed to the glycosylated heavy chains of the HECD-1 antibody used for immunoprecipitation. Treatment of the cells with neuraminidase did not appreciably affect E-cadherin precipitation with HECD-1 (*lanes 4* and *5*), so that it can be assumed that equal amounts of E-cadherin are present in *lanes 2* and *3*. We conclude that sialic acid is a constituent part of E-cadherin on MCF-7/6 cells, and that neuraminidase reduces its presence on that molecule, below the detection limit of the SNA staining method. The SNA staining procedure was checked on Western blots of intact transferrin and desialylated transferrin (not shown).

Sialic acid and E-cadherin mediated cell-cell adhesion. Neuraminidase was used to remove sialic acid from E-cadherin and other molecules on the plasma membrane of MCF-7 cells, and treated and control cells were tested for cell-cell adhesion. The enzyme from Vibrio cholerae can cleave off sialic acid from most of its linkages in glycolipids and glycoproteins, with a preference for 2 → 3 bonds. To check for the efficiency of the neuraminidase treatment in removing sialic acid, cell suspensions obtained by the E-cadherin saving procedure were treated with neuraminidase and the supernatants were assayed for the amount of sialic acid released by the enzyme (Table 2). These amounts were found comparable to the amounts that can be released by the enzyme from other cells. As a reference, we determined the amounts of sialic acid that were set free by treating the cells with 0.05 *M* H_2SO_4. This is considered to represent the total sialic acid of the cell (Table 2). As mentioned above and in Fig. 3, neuraminidase treatment was effective in removing sialic acid from E-cadherin.

Cell suspensions obtained through the E-cadherin saving procedure were treated with neuraminidase and tested in the fast aggregation assay. From Fig. 2 *B*, it can be seen that neuraminidase treatment of the cells abolished their tendency to aggregate. Incubation of MCF-7/AZ cells with IGF-I after the treatment with neuraminidase did not restore aggregation. Incubation of the MCF-7/6 cells with IGF-I after their treatment with neuraminidase did not lead to an induction of cell adhesiveness, as was the case mentioned above with non-enzyme-treated cells (Fig. 2 *D*).

In order to asses whether E-cadherin was preserved on the cell surface after neuraminidase treatment, we treated the MCF-7 cells with the enzyme and then measured their indirect fluorescence after labeling them with RAM-FITC alone, or in combination with the HECD-1 antibody against E-cadherin. From Fig. 4 *C* and *D*, it can be seen that MCF-7/AZ and MCF-7/6 cells still bound the HECD-1 antibody after the enzyme treatment. The peaks were comparable to the peaks obtained with RAM-FITC and HECD-1 on cells that had not been treated with neuraminidase (Fig. 4 *A* and *B*). Preservation of E-cadherin after incubation with IGF-1 has been demonstrated earlier (Bracke et al., 1993).

Pretreatment of the cells with neuraminidase and/or IGF-I did not affect the viability to an noticeable extent. As judged from the explant test, spreading of the cells after 2 h and outgrowth after 24 h did not differ from untreated cells.

Neuraminidase treatment did not inhibit the possibly different, but equally E-cadherin-mediated, process of cell-cell adhesion of MCF-7 cells in the assay for cell scattering. Cells were pretreated with

TABLE 2

AMOUNTS OF SIALIC ACID RELEASED BY NEURAMINIDASE AND 0.05 *M* SULFURIC ACID[a]

	NMOL PER MG OF CELL PROTEIN*	
	MCF-7/AZ	MCF-7/6
A neuraminidase	8.3 (± 1.6)	11.3 (± 1.3)
B 0.05 M H_2SO_4	13.2 (± 0.9)	15.1 (± 4.1)

[a]The cells were detached from their tissue culture plastic substrate by the E-cadherin saving procedure. Approximately 40 × 10^6 cells were washed two times with Ca^{2+}—and Mg^{2+}—free PBS and *A*, The washed cells were resuspended in 1.7 ml of growth medium containing 1% FBS, and treated with neuraminidase. After centrifugation of the cells, the sialic acid in the supernatant was determined with the Warren (1959) method. In the blank, 0.8 ml of acetate buffer was added to 1.7 ml of cell suspension. *B*, The washed cells were treated with 2.4 ml of 0.05 *M* H_2SO_4, and 0.05 *M* H_2SO_4 was used as blank in the sialic acid determination.

For the determination of total cell protein, aliquots of the cell suspensions after their detachment from their substrate were washed and then lysed in a 1% SDS solution at 37°C for 1 h. The lysate was diluted 10 times with distilled water and 0.1 ml of this was taken for determination according Bradford (1976).

* values are means of three separate determinations; ± s.d. between parentheses.

neuraminidase (1 U/10^7 cells) prior to their seeding on plastic substrate, and then grown in their respective media supplemented with a seven times lower neuraminidase concentration as that used during the pretreatment. This did not cause a significant enhancement in the number of solitary cells as assessed after 24 h, nor a widening of the intercellular clefts (Table 1*B*, Fig. 1 *B*). Also addition of neuraminidase to established cultures had no visible effect: subconfluent MCF-7/AZ and MCF-7/6 cells that were growing on tissue culture plastic substrate, were incubated with growth medium containing neuraminidase (1 U/10^7 cells). Visual examination after 45 and 90 min did not reveal significant differences with control MCF-7/AZ cultures that were growing in the same medium without neuraminidase. No increase in the width of the intercellular clefts was observed.

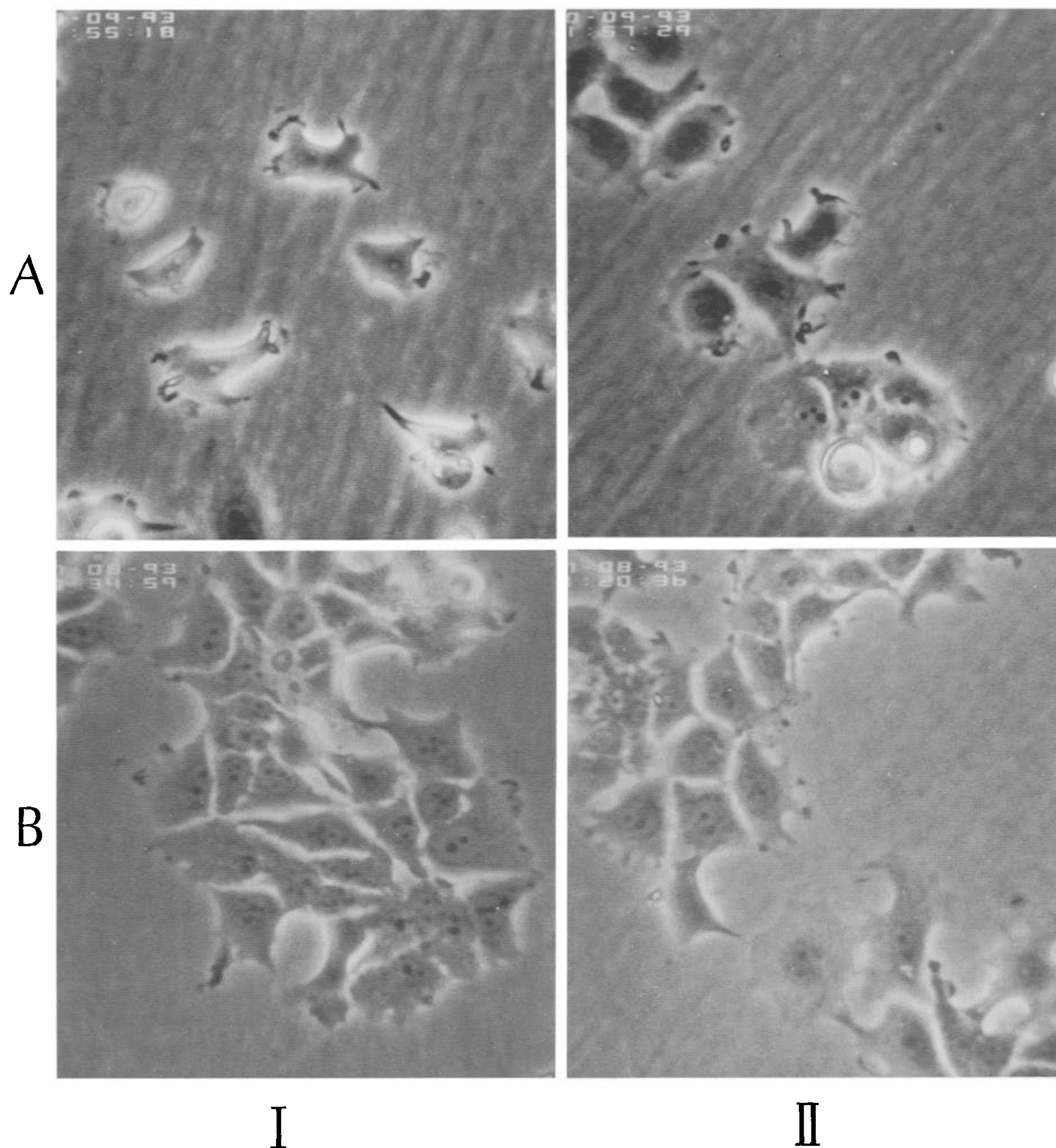

FIG. 1. The cell-scattering effect of MB2 antibody and of neuraminidase on MCF-7/AZ cells. *A*, Addition of MB2 results in a greatly augmented number of cells that make no intercellular contacts (*I*) when compared to a control culture with hybridoma medium (*II*). *B*, Neuraminidase treatment does not noticeably enhance the number of isolated cells or the width of the intercellular distance between adhering cells (*I*), when compared to a control culture without enzyme (*II*). *See* also the Table 1 legend.

Discussion

This study confirms the role of E-cadherin as the dominant cell-cell adhesion molecule on the surface of human breast carcinoma cells (Gamallo et al., 1993; Bracke et al., 1993). Aggregation of suspended MCF-7/AZ cells was found to be completely inhibited by a specific antibody against E-cadherin. The same was found with suspended cells from the MCF-7/6 variant subline that require preincubation with IGF-1 to induce aggregation. It can be concluded that the adhesive bonds responsible for holding the cells together as aggregates that are counted in the aggregation assay are the homophilic E-cadherin bonds. Cells from both cell lines that were grown on a plastic substrate showed a large reduction in the formation of adhesive cell-cell contacts in the presence of an E-cadherin antibody.

In this paper, we report that E-cadherin from MCF-7/6 cells contains sialic acid. Evidence for the presence of sialic acid in E-cadherin from liver cells has been presented by Cunningham et al. (1984). We demonstrate that neuraminidase, which liberates cell surface sialic acid from suspended MCF-7 cells, abolishes their fast aggregation mediated by E-cadherin. In contrast, the enzyme did not affect cell-cell adhesion as measured in the cell scattering assay. Unlike the fast aggregation assay, this assay assesses cell-cell adhesion between cells that are spread on a flat substrate, and this 24 h after seeding of the cell suspension.

We adopted as a working hypothesis that the strength of the adhesive interaction between E-cadherin molecules could be modulated by the presence of sialic acid on these molecules. E-cadherin on MCF-7 cells was found in this study to contain sialic acid, and a substantial part of it was removed by the action of neuraminidase. It seems plausible to ascribe the effect of neuraminidase on suspended cells to a reduction of the amount of sialic acid on E-cadherin. However, the absence of an effect of neuraminidase on spread cells should be explained. Because the enzyme also exerts its action on other glycoproteins and on glycolipids of the cell surface, alternative

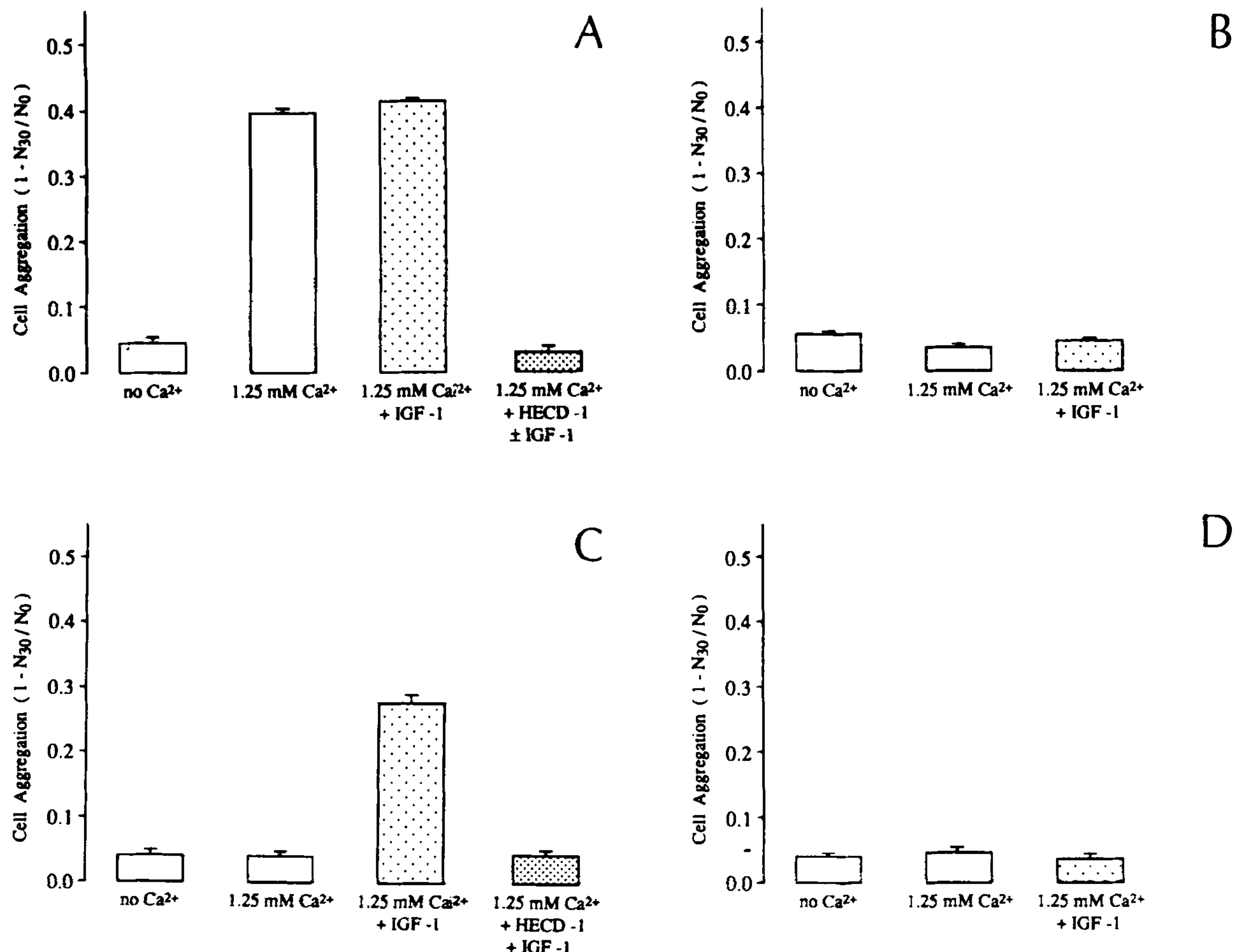

FIG. 2. Adhesion between cells in suspension. The height of the columns is related to the degree of cell aggregation. Mean values from two or three measurements are given. *Horizontal bars* on top of the columns indicate the standard deviations. *A*, MCF-7/AZ cells: The figure shows the calcium dependency of the aggregation and the inhibiting effect of HECD-1 (dilution 1/20). Preincubation with 0.5 μg/ml IGF-I does not affect the adhesiveness. *B*, MCF-7/AZ cells: The cells were treated with neuraminidase before the measurement of the aggregation. *C*, MCF-7/6 cells: The cells do not aggregate in the presence of calcium ions and pre-incubation with 0.5 μg/ml IGF-I is needed. Aggregation is inhibited by HECD-1. *D*, MCF-7/6 cells: Pretreatment with neuraminidase abolishes the stimulatory effect of IGF-I.

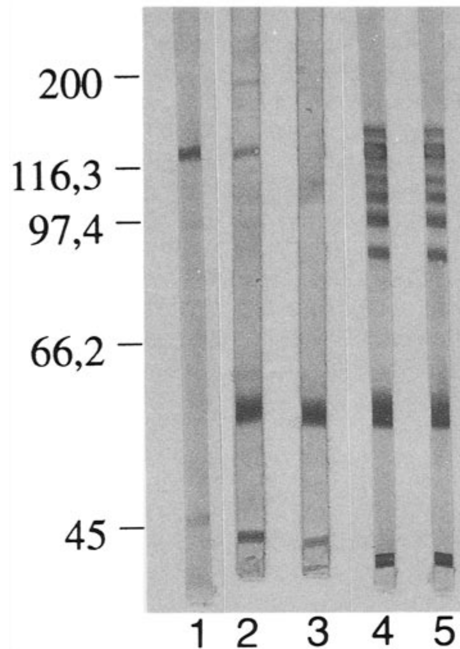

FIG. 3. Immunoblot analysis of E-cadherin in MCF-7/6 cells. Electroblots were made either of the whole cell extract or of the immunoprecipitate obtained with HECD-1. *Lane 1,* Extract of intact cells was stained with HECD-1 antibody against E-cadherin. *Lane 2,* HECD-1 precipitate obtained from intact cells was stained with SNA revealing sialic acid. *Lane 3,* HECD-1 precipitate obtained from neuraminidase-treated cells was stained with SNA. *Lane 4,* HECD-1 precipitate from intact cells was stained with HECD-1. *Lane 5,* HECD-1 precipitate from neuraminidase-treated cells was stained with HECD-1. In *lanes 4* and *5* also breakdown products of E-cadherin are stained with HECD-1.

hypotheses involving indirect effects of sialic acid should be examined.

In contrast to the situation with spread cells, adhesive binding between suspended cells in the aggregation assay is counteracted by shear forces that tend to disrupt the aggregates. A first explanation for the difference of the neuraminidase effect on spread and suspended cells is based on the assumption that the presence of sialic acid moieties on E-cadherin confers additional adhesive strength to the homophilic binding. Cross-linking of the negative charges on E-cadherin molecules on neighboring cells by Ca^{2+}-ions has been proposed by Ringwald et al. (1987). The weaker binding after the release of sialic acid from MCF-7/AZ cells, or from IGF-1-stimulated MCF-7/6 cells is assumed to lead to less stable aggregates in the course of gyratory shaking. These aggregates then are disrupted by the relatively strong shear force exerted during aspiration in the Coulter counter. With cells that are spread on a plastic substrate, shear forces are absent and, therefore, the weaker homophilic E-Cadherin interactions after neuraminidase treatment have still sufficient strength to hold the cells together.

The above explanation seems not to be compatible with the finding of Shirayoshi et al. (1986), who reported that the presence of N-Linked oligosaccharides on the cell surface is not needed for E-cadherin-mediated cell aggregation. E-cadherin probably contains only N-linked carbohydrates (Peyrieras et al., 1985).

Sialic acid also occurs as a constituent of O-linked oligosaccharides and glycolipids. As a second hypothesis, we propose that sialic acids on those molecules are needed for a quick installment of weak intercellular bonds. Electrostatic cross-linking of such sialic acids with cations can provide for quick but weak cell-cell binding. Tetrasaccharides (sialyl-Le^X) on O-linked carbohydrates and glycolipids in which sialic acids are essential elements for adhesive binding have been reported to act as ligands for the selectin type adhesion molecules (Phillips et al., 1990; Springer, 1990; Walz et al., 1990).

In contrast, the installment of the strong homophilic E-cadherin bonds most probably is a relatively slow process. A certain degree of prior cell-cell contact might be needed to initiate the interaction. E-cadherin is a rodlike molecule with a hinge region (Becker et al., 1989), and has been reported to contain specific HAV recognition sequences (Blaschuk et al., 1990). The establishment of an adhesive binding between E-cadherin molecules therefore requires precise juxtaposition. Such juxtaposition is unlikely to be produced solely by random collision of cells.

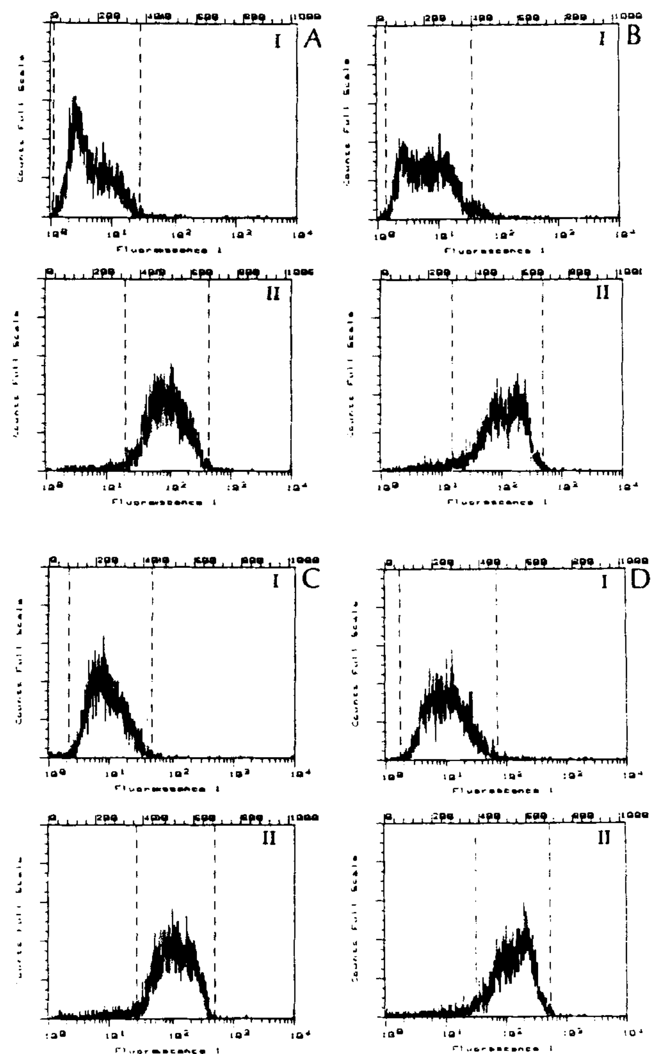

FIG. 4. Flow cytometry of cells labeled with RAM-FITC only (*I*) or in association (*II*) with HECD-1 (dilution 1/20). *A,* MCF-7/AZ cells; *B,* MCF-7/6 cells; *C,* MCF-7/AZ cells pretreated with neuraminidase (1 unit for 10^7 cells); *D,* MCF-7/6 cells pretreated with neuraminidase (1 unit for 10^7 cells).

Whereas spread cells have ample time for the installment of the stable homophilic E-cadherin bonds, with suspended cells that collide during gyratory shaking, the installment of these bonds is supposed to require the transient relative immobilization provided by the weak but quickly installed adhesive forces mentioned above. Removal of sialic acid is thought to diminish weak adhesion and the required relative immobilization. Also, aggregation of cells held together only by weak adhesive forces (i.e., in the absence of functional E-cadherin) is supposed not to be measured by the Coulter counter due to the relatively strong shear force.

A third hypothesis is based on the assumption that sialic acid moieties on the surface of suspended cells are essential elements of the molecular pathway leading to activation of E-cadherin by IGF-I. In line with this is the abolishment of the IGF-1-induced aggregation of MCF-7/6 cells. For MCF-7/AZ cells that aggregate in the absence of added IGF-I, an autocrine IGF-I stimulation should be assumed. To our knowledge, its existence has not been proven.

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