Enlarged Cell-associated Proteoglycans Abolish E-Cadherin Functionality in Invasive Tumor Cells

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Abstract

Mouse and dog epithelial cell lines, expressing high levels of the Ca2+-dependent cell-cell adhesion molecule E-cadherin in vitro, generated invasive and metastatic tumors in athymic mice. From these tumors, neoplastic cell lines were isolated. All ex vivo isolates retained high expression levels of E-cadherin at their surface. Nevertheless, some showed a fusiform morphotype, were defective in Ca2+-dependent cell aggregation, and were invasive in vitro, indicating that E-cadherin was not functional. Cell-associated proteoglycans were found to be enlarged in these variants as compared to their counterparts with functional E-cadherin. Treatment of the cells with the drug 4-methylumbelliferyl β-D-xyloside specifically reduced the amount and size of cell-associated proteoglycans. This same drug induced an epithelial morphotype, increased Ca2+ and E-cadherin-dependent cell aggregation, and abrogated invasiveness without influencing E-cadherin expression levels. Our results indicate that enlarged proteoglycans can prevent the homophilic binding of E-cadherin, probably by steric hindrance. This is one more mechanism by which carcinomas may counteract invasion-suppressor genes and acquire malignancy.

Introduction

Cell-cell adhesive interactions are at the basis of a wide variety of morphogenic events in the vertebrate embryo and adult: These adhesion events are mediated by specific molecules, including the cadherins which are Ca2+-dependent cell-cell adhesion molecules (1, 2). One specific member, E-cadherin, is considered to be a key molecule in the formation of the junctional complex in epithelia. We and others have reported that E-cadherin has a potent invasion-suppressor activity in a variety of epithelial tumor cell types (3–6). Misregulation of E-cadherin during the progression of epithelial tumors to carcinomas can be achieved either by influencing the expression level or, alternatively, by modulating the functionality of the protein. In this paper, we report on a new mechanism by which the functionality of E-cadherin can be abolished, i.e., steric hindrance by large, cell-associated proteoglycans.

Materials and Methods

Cell Lines. The cell lines MDCK-C5 and MDCK-C11 were isolated from the peritoneal cavity of female athymic (nu/nu) mice (Ifa Credo, Brussels, Belgium) 3 weeks after i.p. injection of MDCK-ras-e-pgpt cells, which were either microencapsulated before injection (yielding MDCK-C5) or not (yielding MDCK-C11; [7]). MDCK-ras-e-pgpt is a derivative of a HaMuSV-transformed MDCK cell line (8) expressing an Eco32I-selection gene (7). The cell line NM7-ras was obtained after cotransfection of the cell line NM7, a subclone of the murine mammary gland cell line NMuMG (9), with plasmid pT24 encoding a mutated human c-Ha-ras oncogene and a neomycin resistance marker. NM7-ras cells were injected s.c. into the flank of athymic mice where the developed tumors. Fift-five weeks after injection, animals were killed, and fragments of individual tumor were isolated under sterile conditions. After several washings in PBS, tumor tissue was further cut into pieces of approximately 2 mm3 and shaken at 37°C for 30 min in 0.25% (w/v) trypsin (BDH Chemicals, Poole, United Kingdom) or in PBS supplemented with 0.1% (w/v) collagenase (Boehringer Mannheim, Mannheim, Germany) and 0.125% (w/v) hyaluronidase (Sigma Chemical Company, St. Louis, MO). Segregated cells were then seeded into culture medium supplemented with 800 µg/ml G418 (Gibco Europe, Ghent, Belgium) in order to eliminate host cells from the neoplastic cells. From two individual tumors, we developed the cell lines NM-T3 and NM-T4. The first was obtained after treatment of tumor tissue with collagenase/hyaluronidase and the latter after trypsin treatment.

The cell lines MDC-K5 and MDCK-C11 were grown in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% (v/v) fetal calf serum (Gibco), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. The medium for the cell lines NM-T3 and NM-T4 was identical except for additional supplementation with 10 µg/ml insulin (Sigma).

Reagents. The drugs MUβX (Sigma) and paranitrophenyl-α-xyloside (NBS Biologicals, Hatfield, United Kingdom) were dissolved in dimethyl sulfoxide at a concentration of 500 µg/ml.

Detection of E-cadherin. Immunofluorescence and flow cytometric analysis of E-cadherin were as described (3). In both cases, the primary antibody used was the E-cadherin-specific rat monoclonal antibody DECMA-1 (Sigma). For coimmunoprecipitation of E-cadherin and catenins, cells were labeled for 4 h with 125 µCi [35S]methionine in vivo cell labeling grade (>1000 Ci/mmol; Amersham International, Amersham, United Kingdom) per 10 cm2, washed twice with PBS, and lysed with 500 µl of lysis buffer containing 1% Nonidet P-40–1% Triton X-100 in PBS with Ca2+ and Mg2+. Lysates were cleared and adjusted to equal amounts of trichloroacetic acid-precipitable cpm per volume, and rat monoclonal antibody DECMA-1 (diluted 1:8000) was added. Immune complexes were allowed to form at 4°C for 3 h and then collected using polyvalent rabbit anti-rat IgG (Nordic Immunological Laboratories, Tilburg, the Netherlands) and protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Proteins were eluted and analyzed by sodium dodecyl sulfate gel electrophoresis on 7% gels followed by fluorography.

Aggregation Assay. The Ca2+-dependent cell aggregation assay was performed as described previously (10). Briefly, cells were suspended with trypsin in the presence of 0.04 µg Ca2+ (protecting E-cadherin from proteolytic cleavage) and then allowed to aggregate by gyrotry shaking in the presence of 1 µg Ca2+. The aggregation index was expressed as 1–N30'/N0, where N0 is the initial particle number of the cell suspension and N30' is the number of particles after 30 min of incubation. For MUβX treatment, cells were preincubated for 3 h with the drug and then suspended and assayed as described above, except that MUβX was present throughout the experiments. For treatment with DECMA-1, MUβX-pretreated cells were suspended and preincubated for 30
min at 4°C with the antibody (diluted 1:1000) and then assayed in the presence of DECMA-I and MUβX.

Collagen Invasion Assay. For the collagen invasion assay, cells were seeded on top of a gelified Collagen S (type I) solution (Seromed; Biochrom KG, Berlin, Germany). After 24 h at 37°C, the number of cells that had invaded into the gel was counted (for details, see Refs. 3 and 11). The invasion index is expressed as the fraction (%) of cells that has invaded into the gel over the total number of cells seeded. For MUβX treatment, cells were assayed in the presence of the drug without pretreatment.

Isolation and Separation of Proteoglycans. Cells were labeled for 24 h in regular media supplemented with [35S]sulfate (carrier free, 5 μCi/ml; Amersham). To isolate detergent extracts, cells were washed twice with PBS and lysed in 1% Triton X-100-1% Nonidet P-40 in PBS containing Ca2+ and Mg2+. Lysates were cleared, and the supernatants were adjusted to equal amounts of protein. To isolate trypsin-releasable proteoglycans, cells were washed three times with serum-free medium and then incubated for 20 min at 37°C with an isotonic trypsin-EDTA solution (0.05% trypsin-0.04% EDTA). After neutralization of trypsin with serum, the cell suspension was centrifuged, and the supernatant was collected. The relative mass of the proteoglycans was assessed by Sepharose CL-4B (Pharmacia LKB) chromatography in 4 M guanidinium hydrochloride buffered to pH 5.5 by 50 mM sodium acetate. Radioactivity in solutions was determined by scintillation counting using eight volumes Insta-Gel II (Canberra Packard; Meridian, CT) for each sample volume. Vo and Vt were determined with Blue Dextran 2000 (Pharmacia LKB) and phenol red, respectively.

Results and Discussion

The cell line NM7-ras, a ras-transfected derivative of the murine mammary gland cell line NMuMG (9), showed high in vitro expression of E-cadherin and was injected s.c. into athymic nude mice. From the resulting invasive and metastatic tumors, we isolated two cell lines, designated NM-T3 and NM-T4. Although both derivatives expressed comparable high amounts of E-cadherin at their cell surface (Fig. 1a), the subcellular localization of the protein was different. In NM-T3 cells, which showed an epithelioid morphotype, E-cadherin was concentrated at intercellular contacts (Fig. 1b). In contrast, in
NM-T4 cells, which had a more fusiform morphotype, E-cadherin was less concentrated at the cell-cell borders but was rather spread all over the cell surface (Fig. 1b). Contrary to NM-T3, the NM-T4 cells failed to aggregate in a Ca\(^{2+}\)-dependent and E-cadherin-specific assay (Fig. 2), indicating that E-cadherin was not functional. In previous experiments, functional E-cadherin was shown to counteract invasion (3–6). NM-T4 cells indeed were highly invasive in a collagen type I invasion assay in contrast to NM-T3 cells (Fig. 2).

Similar results were obtained for ras-transformed derivatives of the canine epithelial kidney cell line MDCK (3, 8). Such cells were injected i.p. into nude mice, either as a nonencapsulated cell suspension or after microencapsulation, and after 3 weeks, cell lines were established ex vivo (7). Also in this case, cells either had retained their epithelial morphology (MDCK-C5; obtained from microencapsulated cells) or were much more fusiform (MDCK-C11; obtained from nonencapsulated cells; Fig. 1c). Again, the fusiform morphology was not due to a decrease in the expression of E-cadherin since both cell lines expressed comparable levels at their cell surface (Fig. 1a). Contrary to the epithelioid MDCK-C5 cells, the fusiform MDCK-C11 cells often displayed E-cadherin all over their cell surface (Fig. 1c). Moreover, the MDCK-C11 cells showed no Ca\(^{2+}\)-dependent aggregation (Fig. 2) and were invasive in a collagen type I gel (Fig. 2; Ref. 7). All these data point to the loss of E-cadherin functionality in MDCK-C11 cells also.

Convincing evidence exists that the association of cadherins with particular cytoplasmic proteins, designated catenins, is a prerequisite for E-cadherin functionality (12–14). E-cadherin molecules in the different tumor derivatives were immunoprecipitated using nonionic detergents in order to preserve cadherin-catenin interactions. No differences were found between the proteins that coimmunoprecipitated with E-cadherin in NM-T3 versus NM-T4 cells on the one hand and in MDCK-C5 versus MDCK-C11 cells on the other hand (Fig. 1d). These coimmunoprecipitating molecules included proteins with a molecular mass of 102, 88, and 80 kDa, probably corresponding to α-, β- and γ-catenin, respectively (13). Hence, it seems unlikely that changes in association of E-cadherin with cytoplasmic proteins are responsible for the observed loss of functionality.

Subsequently, we wondered whether the synthesis of proteoglycans was altered in the different cell types. Radiolabeled proteoglycans were isolated either as detergent extract or after trypsin treatment of intact cells. Evaluation by gel filtration chromatography of the amount and size of the cell-associated proteoglycans revealed that these molecules were enlarged in NM-T4 versus NM-T3 cells (Fig. 3a) and in MDCK-C11 versus MDCK-C5 cells (Fig. 3b).

In order to assess whether reduction of the proteoglycan size could induce the functionality of E-cadherin, NM-T4 and MDCK-C11 cells were treated with MUBX. This drug serves as a synthetic primer for the synthesis of GAG chains and competitively inhibits the anchorage of GAG side chains to the proteoglycan core proteins (15, 16). For NMuMG cells, 0.5 mM of MUBX was reported to prevent only the addition of chondroitin sulfate side chains to the core proteins, whereas at 5 mM, also heparan sulfate side chains are prevented to attach (17). Treatment of NM-T4 and MDCK-C11 cells with 5 and 0.5 mM of MUBX, respectively, enhanced the intercellular contacts within 2 h. The morphology of the treated cells resembled the epithelioid one of NM-T3 and MDCK-C5, respectively (Fig. 1, b and c). The morphological effects of MUBX were completely reversible after removal of the drug. Immunofluorescence analysis revealed that these changes were associated with redistribution of E-cadherin to a honeycomb pattern, resembling the localization present in NM-T3 and MDCK-C5 cells (Fig. 1, b and c), whereas flow cytometric analysis showed no influence of MUBX on E-cadherin levels at the cell surface (illustrated for MDCK-C11 cells in Fig. 4a). MUBX did reduce both amount and size of cell-associated proteoglycans (illustrated for MDCK-C11 cells in Fig. 4b). Induction of E-cadherin functionality by MUBX was proven by the fact that drug-treated NM-T4 and MDCK-C11 cells showed Ca\(^{2+}\)-dependent aggregation and were unable to invade into collagen gels (Fig. 4c). Induction of cell aggregation by MUBX could be prevented by cotreatment with the E-cadherin-blocking antibody DECMA-1 (Fig. 4c). As an additional control, closely related cell lines NM-Y1 and MDCK-L2 were assayed. These cell lines express the ras oncogene, are tumor derived, but do hardly express E-cadherin. Treatment of these cells with MUBX did not induce an epithelioid morphology or Ca\(^{2+}\)-dependent aggregation or abrogate invasiveness (partly shown in Fig. 4c).

The observed proteoglycan effect may be due to direct steric hindrance with the extracellular domains of E-cadherin molecules. However, since it is well documented that proteoglycans can bind to several growth factors (18, 19), thereby inhibiting or activating their function, alternative explanations for the present results need consideration. Secreted proteoglycans (or other proteins) could be responsible for the modulation of E-cadherin function. This, however, is very unlikely since exchange of conditioned media between the different cell lines had no effect on morphology (data not shown). Also, an indirect effect of MUBX, as a result of enhanced secretion of free GAGs in the medium, could be excluded. When conditioned medium of MUBX-treated cells was dialysed in order to remove MUBX, this medium, still enriched in free GAGs, no longer induced E-cadherin

![Cell Aggr. (1-N30'/N0')](attachment:image.png)  
![Invasion Index (%)](attachment:image.png)

**Fig. 2.** Comparison of Ca\(^{2+}\)-dependent cell aggregation (left) and invasion into collagen type I gels (right) for NM-T3 versus NM-T4 cells and for MDCK-C5 versus MDCK-C11 cells.
Fig. 3. Analysis of cell surface proteoglycans of tumor-derived cell lines. a, sizing chromatography of detergent-extracted [35S]sulfate-labeled proteoglycans of NM-T3 and NM-T4 cells. b, sizing chromatography of trypsin-released [35S]sulfate-labeled proteoglycans of MDCK-C5 versus MDCK-C11 cells. Vo, the void volume of the column. The right end of the X-axis corresponds with the total volume of the column (Vt).

functionality in fresh NM-T4 or MDCK-C11 cell cultures (data not shown). Recently, it was reported that both α- and β-xylosides can influence glycolipid synthesis (20), implying that the effects observed in the present paper might be glycolipid and not proteoglycan dependent. However, treatment of NM-T4 and MDCK-C11 cells with, respectively, 5 and 0.5 mM paranitrophenyl-α-xyloside, a drug that influences glycolipid but not proteoglycan synthesis, had no effect on morphotype or Ca2+-dependent cell aggregation (data not shown).

In conclusion, the results described here demonstrate that large cell surface proteoglycans can inhibit the functionality of normal levels of E-cadherin. Steric hindrance by such large and highly charged proteoglycans may be the direct and major cause for this effect by preventing the homophilic and homotypic binding of E-cadherin molecules between adjacent cells. A similar mechanism has been suggested for mucins, which are also very large and highly charged cell surface molecules (21). Steric hindrance by proteoglycans has so far only been described to interfere with cell-matrix adhesion (22, 23). Considering the tumoral nature of the cell lines tested, one may speculate that neoplastic cells in a malignant epithelial tumor use the synthesis of large proteoglycans as one of the mechanisms to overcome the invasion-suppressive function of E-cadherin.

Presently, we have no evidence that one specific family of cell-surface proteoglycans is involved. We rather believe that, depending on the cell type, various cell-associated proteoglycans can be enlarged, all resulting in interference with cadherin functionality. Other mechanisms to suppress the activity of E-cadherin are the down-regulation of its expression (24, 25) and the acquisition of particular mutations and defects in E-cadherin or in the catenins (26, 27). Our findings suggest an explanation for the malignant properties of various human carcinoma types with cells showing loss of polarization despite high levels of E-cadherin (2, 28-33). In such carcinoma cells, E-cadherin often shows aberrant subcellular localization. In consequence, it might be most rewarding to further analyze the influence of proteoglycan-specific drugs on the malignant properties of animal and human tumors. Moreover, it is conceivable that comparable mechanisms for cadherin inactivation are used in a more controlled way.

Fig. 4. Effects of MUBX on various cell properties. a, flow cytometric analysis of E-cadherin expression in untreated MDCK-C11 cells versus cells treated with 0.5 mM MUBX for 3 h. In the controls (dashed lines), the primary antibody was omitted. b, sizing chromatography of trypsin-released [35S]sulfate-labeled proteoglycans of untreated MDCK-C11 cells versus cells treated with 0.5 mM MUBX for 3 h. c, Ca2+-dependent cell aggregation (left) and invasion into collagen gel (right) of malignant cells in the absence or presence of MUBX and E-cadherin-blocking antibody DECMA-1. The Ca2+-dependent aggregation of murine cell lines NM-T4 and NM-Y1 and of canine cell lines MDCK-C11 and MDCK-L2 was performed with the cells either untreated (open columns ± SEM) or assayed in the presence of MUBX (solid columns ± SEM). In both the aggregation and invasion assays, the concentration of MUBX was 5 mM for NM-T4 and NM-Y1 and 0.5 mM for MDCK-C11 and MDCK-L2.

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during normal morphogenesis or wound healing, where migration of epithelial cells is warranted.

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References

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