Involvement of the cell surface-bound mucin, episialin/MUC1, in progression of human carcinomas

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Introduction

Episialin is a major sialylated glycoprotein at the apical surface of exocrine glandular cells and throughout the entire surface of many types of carcinoma cells. Numerous monoclonal antibodies (mAbs) have been raised against this highly sialylated and sulphated glycoprotein from carcinoma cells and milk fat globule membranes [1-5]. It is a membrane-bound glycoprotein that can be released from the cell surface by an unknown mechanism. In patients with breast and other carcinomas, episialin is shed from the cancer cells into the circulation and can be detected by the CA 15-3 assay, which is presently being used for monitoring the course of the disease and for early detection of recurrent breast cancer [6,7]. The assay uses an mAb, 115D8, developed by our group [8] and mAb DF3 raised by Kufe and colleagues [9].

Episialin is synthesized as a large single polypeptide, in most cell lines approximately 200 kDa or more [10,11]. This precursor is rapidly cleaved by proteolysis into a small moiety containing the transmembrane and cytoplasmic domains and a larger part that constitutes most of the extracellular domain. Both moieties remain non-covalently associated [12]. This proteolytic processing step occurs in the endoplasmic reticulum and may be essential for further maturation. The main processing of episialin occurs by the addition of numerous O-linked glycans, which increases the apparent molecular mass on SDS-polyacrylamide gels to more than 400 kDa. The extensive glycosylation protects the molecule against proteolytic degradation, since the non-O-glycosylated precursors are degraded rapidly, whereas the mature molecule is extremely resistant to the action of proteases. The last step in the processing of episialin is the addition of sialic acid residues to the glycans, which increases the mobility of the molecule on SDS gels.

The early proteolytic cleavage step is not directly responsible for the release of episialin from the membrane, suggesting that episialin is most likely released from the membrane by a second proteolytic cleavage step after arrival at the cell surface. The latter proteolytic cleavage seems to be a slow and might be a random process, allowing the mucin to remain associated with the cell surface with a half-life of 16-24 h.

As a result of differential glycosylation, there are many different glycoforms of episialin. Some glycoforms are preferentially present on carcinoma cells and others are preferentially present on one or a limited number of tissues [5,13-15].

As determined with mAbs that are directed against a non-glycosylated, non-repeat region of the molecule, and by RNA in situ hybridization, the expression of the molecule is strongly increased in most carcinoma cells relative to the corresponding normal epithelial cells. For example, we have found that the expression level in primary breast cancer cells is at least 10-fold higher than the level in normal breast epithelium. The biological background for the up-regulation of episialin expression has not yet been determined.

In this report, we will briefly give an outline of the structure of the molecule, we will show the effect of this elongated molecule on cellular adhesion and metastasis and discuss its possible role in maintaining the integrity of the ducts.

Structure of episialin

We and several other groups [16-19] have cloned episialin cDNA. Subsequent sequence analysis has revealed that episialin is synthesized as a transmembrane molecule with a relatively large extracellular domain and a cytoplasmic domain of 69 amino acids. The extracellular domain mainly consists of a region of nearly identical repeats encoding 20 amino acids. The number of repeats is highly variable in the human population, leading to substantial differences in molecular weights of the episialin molecules from different individuals [20]. The repeats, together with adjacent degenerated repeats, contain many serines and threonines, which are potential attachment sites for O-linked glycans and constitute a mucin-like domain that comprises more than half of the polypeptide backbone, even in the smallest allele detected. The number of tandem
repeat sequences in each allele can vary from approximately 30 to 90.

Twenty per cent of the amino acids in the protein backbone of the mucin domain are prolines, which induce many β-turns [21] and prevent α-helix formation, resulting in a molecule with an extended structure [22]. The extended structure is very rigid as a result of the numerous O-linked glycans attached to the molecule. According to Jentoft [22], an extensively O-glycosylated polypeptide of 20 amino acids is approximately 5 nm long. This means that the mucin-like domain of episialin extends 200–500 nm above the cell membrane [23]. Electron microscopic analysis of purified episialin molecules as well as episialin presented on an in vitro cell line has revealed that episialin indeed has the predicted elongated thread-like structure. Figure 1 shows a model of episialin.

Episialin is unlike the genuine mucins that are secreted by specialized cells and form the mucus layer in, for example, the gastrointestinal tract. The genuine mucins are secreted by highly specialized cells such as the goblet cells of the intestine or the mucus-producing salivary glands, and form gels covering large areas. Before secretion, they are stored intracellularly in storage vesicles, where they form large oligomers linked by S–S bonds [24]. After secretion the tangled polymers do not remain bound to the cell surface. In contrast, cell surface-bound mucins, such as episialin, CD43 and epiglycanin, are anchored in the membrane and are not produced by a highly specialized cell type. They are not secreted but are shed from the cell surface. These mucins do not form gels. In Figure 1 both types of mucins are depicted schematically. The common characteristic of both types of molecules is that a large part of the protein backbone consists of an array of threonine-, serine- and proline-rich tandem repeats that are heavily O-glycosylated. This region, which in secreted mucins is flanked by cysteine-rich sequences, constitutes the actual mucin domain.

**Episialin expression and cellular adhesion**

To investigate the effect of overexpression of episialin on tumour cells we have transfected several cell lines with episialin cDNA under the control of the cytomegalovirus (CMV) immediate early promoter. A proportion of the cells of several of the transfected cell lines were growing in suspension (Figure 2), whereas the adhesion of control cells was not affected, indicating that cell–matrix interactions were reduced. Indeed, adhesion of the transfectants to individual extracellular matrix components such as laminin, fibronectin and collagen I and IV was also strongly affected, confirming the notion that episialin reduces the integrin-mediated adhesion [25]. Episialin-negative revertant cells, which were bulk selected with the cell sorter, were used as controls.

In addition to the reduced cell–matrix adhesion, the transfected cells also exhibit a reduced cell–cell adhesion as compared with the revertants, as was shown in cell aggregation assays [26]. In fact, overexpression of episialin on one of two interacting cells is sufficient to inhibit aggregation.

As we have discussed above, episialin towers 200–500 nm above the plasma membrane, whereas most proteins at the cell surface remain inside the boundaries of the glycocalyx, which is approximately 10 nm thick. Therefore, we have tested whether the size of the molecule is responsible for the anti-adhesion effect of epi-
Episialin interferes with immune recognition

As discussed above, episialin prevents cell–cell adhesion, therefore we assumed that the molecule may also prevent conjugate formation between cytotoxic effector cells and their target cells. Indeed, episialin-transfected A375 melanoma cells were unable to form conjugates efficiently with recombinant interleukin-2 (rIL-2)-activated large lymphocytes [lymphokine-activated killer (LAK) cells] and allogeneic cytotoxic T lymphocytes (CTLs), stimulated with A375 cells, whereas conjugate formation between the lymphocytes and the revertants, which had lost episialin expression, was hardly affected. Subsequently, we have measured the lysis of episialin-transfected A375 melanoma cells and episialin-negative revertants by the LAK cells and CTLs with time, in a 51Cr-release assay. The kinetics of lysis of episialin-negative cells by the LAK cells was similar to that of K562 cells, the standard target of LAK cells, whereas lysis of the episialin-positive A375 transfectants was significantly slower [27]. However, the maximal percentage of target cells lysed eventually reached similar levels. Nevertheless, the slower rate of killing of episialin-expressing cells might be crucial to the survival of metastasizing cells.

Episialin expression and experimental metastasis

We have injected an episialin-transfected A375 clone and revertants of these cells into the tail vein of 21 to 28-day-old Balb/c nu/nu mice. The mice were killed after 5–7 weeks, and the number of tumour nodules in the lungs was counted. The number of nodules in the lungs from the mice injected with the episialin-positive A375 cells was significantly higher than in lungs from mice injected with revertant cells. In some animals injected with revertant cells we observed a relatively large number of metastases. About 25% of these metastases turned out to be positive for episialin, whereas fewer than 1% of the
injected cells were episialin positive as shown by FACScan analysis, suggesting that episialin-positive cells have a higher propensity to metastasize. Other tissues were only rarely affected (as determined at the macroscopic and microscopic level), with the exception of the brain. We conclude that episialin-positive cells metastasize more efficiently to the lung than episialin-negative cells. Since episialin protects cells against immune attack by LAK cells (closely related to natural killer cells, which form the main cellular immune defence of nude mice) in vitro, it is tempting to speculate that the difference in the formation of experimental metastases is due to escape of the episialin-positive cells from immune destruction. Alternatively, the differences in adhesion properties of the episialin transfectants and revertants may cause the difference in metastatic potential between both cell types.

Conclusions
Episialin overexpression in tumour cells strongly diminishes the adhesive properties of the cell. In normal polarized cells, episialin is only present at the apical side of the cell, and it is not expected to interfere with adhesion. However, tumour cells often lose their normal polarized architecture, and as a consequence episialin will be present in the same domains as adhesion molecules. This will lead to interference with cellular adhesion, provided the expression level of the membrane-bound mucin is sufficiently high. As we have shown here, this has important implications for biological processes involving cell adhesion, such as cytotoxic T-cell activity and metastasis. The levels of episialin on metastatic breast, ovarian and other carcinoma cells in pleural or ascites fluids from patients are similar to or even higher than those on the A375 and other transfectants discussed above (J. Hilkens, unpublished work), indicating that episialin may also interfere with adhesion or processes involving adhesion of metastatic cells in vivo.

To maintain the functional properties of normal ducts, it is important that the opposite apical domains of the ductal epithelial cells do not adhere to each other as a result of non-specific protein–protein interactions or the presence of bona fide adhesion molecules, such as CEA, that might be found at the apex of the cell. We have shown above that episialin can prevent cell–cell and cell–matrix adhesion of cultured cells. In normal glandular ductal cells episialin is present at the apical side, therefore it seems likely that episialin has a similar anti-adhesive function in the apical domain of these cells, and will help to prevent non-specific adhesion of opposite apical domains of ductal cells, provided the regional density of the molecule is sufficiently high. However, Gendler et al. [28] have generated mutant mice that lack episialin expression. These mice showed no phenotype and the development of normal ductal structures, suggesting either that episialin is not needed for maintaining integrity of the ducts or that there is sufficient redundancy provided by other cell surface-bound mucins of the mouse such as epiglycanin [29].

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MUC1, endometrium and embryo implantation
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Structure of MUC1 and its variants
MUC1 is a large type 1 cell-surface glycoprotein with a short cytoplasmic tail (69 residues) and a large ectodomain containing a variable number tandem repeat (VNTR) sequence [1-4]. The TR is a sequence of 20 amino acid residues with five potential O-glycosylation sites (serine or threonine), of which at least two threonine residues appear to be used [5]. The number of repeats present varies from about 20 to 80, with individuals normally expressing two co-dominant alleles of different size. In addition to O-glycosylation, there are also N-glycosylation sites in the membrane-proximal region. The molecular mass of the mature product varies in the range 200-500 kDa.

The cell-surface form of MUC1 is targeted to the apical surface of epithelial cells in many tissues [6-13]. The cytoplasmic domain may be phosphorylated [14]. In certain carcinomas MUC1 expression is elevated and the polarity of its cell-surface distribution is lost [6-8, 15-17].

In addition to the full-length product there are two known variant forms of MUC1. One is a short transmembrane form known as MUC1/Y [18] that arises by alternative splicing of mRNA. It lacks the TR and contains an extracellular domain of only 134 amino acid residues. It gives rise to products of 33 and 42 kDa, which probably represent different glycoforms.

The second variant form is a secreted glycoprotein that lacks the transmembrane and cytoplasmic sequences of MUC1 but contains the full-length VNTR. This has been detected in medium conditioned by carcinoma cell lines, where it has been suggested to arise by proteolysis and release from the cell surface [19,20]. However, there are also data to suggest that a secreted variant might arise by mRNA splicing [4,21]. This product could account for the presence of MUC1 in cancer serum [22]. It is not known what role this product plays in normal epithelial physiology.

Expression of MUC1 variants in endometrium and endometrial carcinoma cell lines
During the menstrual cycle, endometrium shifts from a regenerative phase of proliferation under oestrogen stimulation to a secretory phase in which differentiation occurs as a result of the combined action of progesterone and oestrogen

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