Episialin (MUC1) Inhibits Cytotoxic Lymphocyte-Target Cell Interaction

Elly van de Wiel-van Kemenade,* Marjolijn J. L. Ligtenberg,† Annemiek J. de Boer,* Femke Buijs,† Hans L. Vos,† Cornelis J. M. Melief,* John Hilkens† and Carl G. Figdor3*

*Division of Immunology and †Division of Tumor Biology, The Netherlands Cancer Institute, Antoni van Leeuwenhoek Huis, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

ABSTRACT. Episialin (MUC1) is a mucin-like glycoprotein abundantly expressed on most carcinoma cells. As a result of its extended and rigid structure, it reduces intercellular adhesion. We investigated whether this antiadhesion function allows tumor cells expressing high levels of episialin to escape from immune recognition. To test this hypothesis, we transfected episialin-negative (episialin-) melanoma cells (A375) with the MUC1 cDNA-encoding episialin. The results demonstrated that episialin-positive (episialin+) melanoma cells were significantly less susceptible to lysis than episialin- melanoma cells by both alloantigen or rIL-2-stimulated cytotoxic effector cells. In addition, cold target inhibition experiments with episialin+ and episialin- cells clearly demonstrated preferential lysis of episialin- cells. Furthermore, antibody blocking studies showed that lysis of episialin+, but not of episialin-, melanoma cells was predominantly dependent on the leukocyte function-associated Ag-1/intracellular adhesion molecule adhesion route, suggesting that episialin- target cells adhere less efficiently to effector cells than episialin+ target cells. This notion was supported by the observation that conjugate formation of the effector cells with episialin+ target cells was significantly impaired. From these results we conclude that over-expression of episialin as found on many tumor cells may indeed affect efficient lysis by cytotoxic lymphocytes and thus may contribute to escape from immune surveillance.

Episialin is a transmembrane, mucin-like glycoprotein encoded by the MUC1 gene. It is mainly present on glandular epithelial cells, and its expression is substantially increased in many carcinomas (1). Full-length cDNA encoding episialin has been cloned and sequenced. The large extracellular domain consists mainly of repeats of 20 amino acids (2–5). As a result of a genetic polymorphism, the number of these repeats varies between about 30 and 90, causing a variation in length of the protein between 1000 and 2200 amino acids. Episialin contains many proline, serine, and threonine residues and numerous O-linked carbohydrate side chains (2–5). These characteristics indicate that episialin has a rigid structure and extends far above the plasma membrane, and thus, if present in sufficient quantity, this cell surface molecule would effectively shield the cells from interactions with other cells and macromolecules (6). We previously showed that expression of large amounts of episialin on the cell surface after transfection with episialin cDNA prevents homotypic aggregation of melanoma or mammary epithelial cells. Similarly, aggregation of episialin- cells with episialin+ mammary epithelial cells is disturbed (7). In contrast to this antiadhesion effect of episialin, it has also been shown that episialin can elicit a specific cytotoxic T cell response (8). It was suggested that this is the result of a differential glycosylation of the repeated structure of the molecule. This repeated epitope can be recognized by TCR in a MHC non-restricted manner (8, 9).

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2 Present address, Department of Immunohaematology and Bloodbank, Academic Hospital Leiden, Rijnburgerweg 10, 2333 AA Leiden, The Netherlands.

3 Address correspondence and reprint requests to Dr. C. G. Figdor, Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

4 Abbreviations used in this paper: episialin+, episialin-positive; episialin-, episialin-negative; LAK cells, lymphokine activated killer cells; ICAM, intracellular adhesion molecule; LFA, leukocyte function-associated Ag.
Despite the fact that episialin can induce a TCR-mediated immune response, the antiadhesion function of episialin has led to the hypothesis that high expression of episialin on tumor cells may also result in escape from the immune system. Both specific (TCR-mediated) and non-specific (without TCR involvement) cytotoxic activity of lymphocytes require cellular interactions with their target cells (10). These cellular interactions are not only mediated by the TCR/CD3 complex but also by a number of adhesion molecules, including CD4, CD8, CD2, and LFA-1, which bind to their respective ligands, MHC class II, MHC class I, LFA-3, and ICAM-1 (11).

In the present study, we investigated whether expression of high levels of episialin by target cells, will affect their ability to be recognized by cytotoxic lymphocytes. If episialin indeed impairs cell-cell contact, this may have important consequences for the survival of tumor cells in the circulation, and may thus contribute to the metastatic process. We, therefore, transfected the episialin+ human melanoma cell line A375 with cDNA-encoding episialin and tested both episialin+ and episialin− melanoma cells for their susceptibility to nonspecific lysis by lymphokine-activated peripheral blood lymphocytes, or to TCR/CD3-mediated lysis by T lymphocytes, alloantigen stimulated in mixed lymphocyte-tumor cell culture with A375 melanoma cells.

**Material and Methods**

**Cell lines and cell cultures**

Mononuclear cell suspensions of healthy volunteers were fractionated by centrifugal elutriation as described previously (12). The cell fraction containing T lymphocytes (CD2+, CD3+) and NK cells (CD2−, CD3−) was cultured for 5 days in Iscove's medium supplemented with 5% human serum, 1% antibiotic/myotic solution (Flow, Irvine, Scotland) and 0.2% kanamycin solution (Sigma, St. Louis, MO), and stimulated with 500 IU/ml rIL-2, kindly provided by Eurocetus (Amsterdam, The Netherlands) before they were used as LAK cells. This LAK cell population contained approximately 20% CD3−, CD2+, CD56+, and CD57+ cells (NK cells). Table I shows the phenotype of a representative LAK cell population.

Freshly isolated allogeneic PBL (1 × 10⁶/ml), mixed with 1 × 10⁵/ml irradiated A375 melanoma cells (100 Gy) were maintained in Iscove's medium supplemented with 5% human serum, antibiotic/myotic, and kanamycin. The PBL were restimulated, once a week, with irradiated A375 cells to generate an allospecific response. In addition, after 3 wk of culturing, rIL-2 (100 IU/ml) was added weekly, and after 4 wk, also a mixture of feeder cells [irradiated (50 Gy) allogeneic EBV-transformed B cells and irradiated (40 Gy) allogeneic PBL, stimulated with 0.2 µg/ml phytohemagglutinin] was added biweekly to further stimulate growth of effector cells. After 8 wk of stimulation, the lymphocyte culture had acquired a stable phenotype and was used as effector cells (cytotoxic T lymphocytes). All CTL expressed the αβTCR, and the main population was CD8+ (Table I). Both effector cells (LAK cells and CTL) were LFA-1+, LFA-3+, and ICAM-1+. The expression levels of these Ag were comparable on both type of effector cells.

The melanoma cell lines A375 (13), MeWo (14) and 518A2 (15), and the MHC-negative, LAK-sensitive prostate leukemia cell line K562 were maintained in Iscove's medium supplemented with 10% FCS, antibiotic/myotic, and kanamycin. All cell lines were free of mycoplasma contamination.

<table>
<thead>
<tr>
<th>Ag</th>
<th>mAb</th>
<th>Positive Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>CLB-T11/1</td>
<td>94</td>
</tr>
<tr>
<td>CD2</td>
<td>CLB-T11/1</td>
<td>94</td>
</tr>
<tr>
<td>CD3</td>
<td>CLB-T3/3</td>
<td>74</td>
</tr>
<tr>
<td>CD4</td>
<td>BMA031</td>
<td>73</td>
</tr>
<tr>
<td>CD56</td>
<td>Leu 19</td>
<td>21</td>
</tr>
<tr>
<td>CD57</td>
<td>Leu 7</td>
<td>14</td>
</tr>
<tr>
<td>CD16</td>
<td>CLB-FcGr/1</td>
<td>32</td>
</tr>
<tr>
<td>CD20</td>
<td>B1</td>
<td>6</td>
</tr>
</tbody>
</table>

**Transfection and generation of revertant A375 melanoma cells**

Cloned A375 cells (A375B, further referred to as A375 cells) were transfected with the expression vector pCMVIE-AK1-DHFR (16), in which the coding domain of episialin cDNA was inserted, and the DHFR gene was removed (7). The vector contains a neomycin-resistance gene. Transfections were performed using the calcium-phosphate co-precipitation method (5). After 3 wk, neomycin-resistant clones were isolated and the expression of episialin was examined by FACScan (Becton Dickinson, Mountain View, CA) analysis as described previously (17). To generate revertant melanoma cells, episialin+ A375 cells were cultured in the absence of neomycin. Episialin− cells were 2 to 4 times sorted by FACS tar (Becton Dickinson, Mountain View, CA) as described previously (17). To generate revertant melanoma cells, episialin+ A375 cells were cultured in the absence of neomycin. Episialin− cells were 2 to 4 times sorted by FACScan (Becton Dickinson, Mountain View, CA) as described previously (17). To generate revertant melanoma cells, episialin+ A375 cells were cultured in the absence of neomycin. Episialin− cells were 2 to 4 times sorted by FACScan (Becton Dickinson, Mountain View, CA) as described previously (17). To generate revertant melanoma cells, episialin+ A375 cells were cultured in the absence of neomycin. Episialin− cells were 2 to 4 times sorted by FACScan (Becton Dickinson, Mountain View, CA) as described previously (17).
The following mAb were used for phenotypic analysis and for functional inhibition studies. 139H2 (anti-episialin) was generated as described previously (18). The following mAb were kindly provided by several laboratories: CLB-LFA-1/1 (anti-LFA-1 common β chain, CD18) (19) (Dr. F. Miedema, Amsterdam, The Netherlands); CLB-T11/1 (anti-CD2) and CLB-T3/3 (anti-CD3) (20) (Dr. R. A. W. van Lier, Amsterdam, The Netherlands); F10.2 (anti-ICAM-1, CD54) (21) (Dr. A. C. Bloem, Utrecht, The Netherlands); RIV-6 (anti-CD4) (22) (Dr. F. UytdeHaag, Bilthoven, The Netherlands); TS2/9 (anti-LFA-3, CD58) (25) (Dr. T. A. Springer, Boston, MA); W6/32 (anti-MHC class I) (26) (Dr. W. J. M. Tax, Nijmegen, The Netherlands); CLB-Fc-Gr/1 (anti-CD20) (28) (Coulter Corporation, Hialeah, FL); Leu 7 (anti-CD3) (19) (Dr. R. Kurrel, Marburg, Germany); B1 (anti-CD20) (28) (Coulter Corporation, Hialeah, FL); Leu 7 (anti-CD57) (29) and Leu 19 (anti-CD56) (30) (Becton Dickinson, San Jose, CA).

Immunofluorescence
Cells were incubated (30 min, 0°C) in PBS, containing 0.5% wt/vol BSA (Sigma), and 0.02% wt/vol sodium azide, with appropriate dilution of the different mAb, followed by incubation with FITC-labeled goat F(ab')2 anti-mouse IgG antibody (GAM-FITC, Nordic, Tilburg, The Netherlands) for 30 min at 0°C. The relative fluorescence intensity was measured on a FACScan (Becton Dickinson).

Cytotoxicity assay
The 1 × 10^3-labeled target cells (100 μCi [51]Cr/10^6 cells, Amersham, Buckinghamshire, UK) were placed in each well of V-bottom microtiter plates with varying numbers of effector cells in 150 μl Iscove's medium with 0.25% BSA. The plates were centrifuged for 5 min at 50 g and incubated at 37°C and 5% CO₂. After the incubation period, 100 μl of supernatant was collected from each well and counted in a gamma counter. Results were expressed as the mean percentage of cell binding from triplicate wells.

Results
Episialin expression by A375, transfectants and revertants
We have generated three sets of episialin-transfected cell lines (ACA19D4⁺, ACA25F8⁺, ACA30D8⁺) and revertants of these lines, which have lost the episialin expression (ACA19D4⁻, ACA25F2⁻, ACA30D8⁻). Immunofluorescence analysis showed a high level of episialin expression on transfected A375 melanoma cells (ACA19D4⁺, ACA25F8⁺, ACA30D8⁺), whereas parental (A375) and revertant melanoma cell lines (ACA19D4⁻, ACA25F2⁻, ACA30D8⁻) expressed no episialin on their cell surface (Table II). The level of episialin expression by transfected cells was comparable to that observed on carcinoma cell lines (data not shown). Furthermore, the m.w. of the precursor and mature forms of episialin expressed by the transfectants were comparable to that of the T47D cells, the cell line from which the cDNA had been derived (7). Further phenotypical analysis of cell surface expression of ICAM-1, LFA-3, and MHC class I molecules involved in cytolytic interaction revealed that all cell surface markers tested were expressed as well as on the parental cell line A375 as on the episialin-transfected and -reverted cell lines,
although the levels of expression were lower on episialin+ cells (Table II). This is probably due to the fact that high episialin expression affects accessibility of the antibodies to their respective Ag, since capping of episialin on the cell surface diminished this effect (data not shown).

Lysis of episialin transfected melanoma cells

We investigated the susceptibility to lysis of episialin-transfected melanoma cell lines (ACA19D4+, ACA25F8+, ACA30D8+), reverted melanoma cell lines (ACA19D4−, ACA25F2−, ACA30D8−), the parental cell line (A375), two unrelated melanoma cell lines (MeWo and 518A), and the NK- and LAK-sensitive target cell line (K562). To this end, we used two types of effector cells: 1) alloantigen stimulated CTL and 2) LAK cells. The specificity of both effector cells is illustrated in Table III. The CTL lysed the melanoma cell lines used as allogeneic stimulator cells, whereas two unrelated melanoma cell lines and K562 were not lysed. In contrast with CTL, LAK cells lysed all target cell lines (Table III). Furthermore, the involvement of the TCR/CD3 complex in lysis by CTL is demonstrated by addition of anti-CD3 mAb that significantly inhibited lysis by CTL (48% to 66%), whereas this mAb was incapable of inhibiting nonspecific lysis by LAK cells (Table III).

In addition, we studied the susceptibility to lysis of episialin+ and episialin− cells after various incubation times. The results in Figure 1A–C show that episialin+ melanoma cells were less susceptible to lysis than episialin− melanoma cells by CTL. We also measured the percentage lysis of ACA30D8+ and ACA30D8− by LAK cells. Episialin+ melanoma cells were lysed considerably slower compared to episialin− target cells by LAK cells (Fig. 1D). Although ACA30D8+ cells are less well lysed than the ACA30D8− cells, after 4 h of incubation almost similar amounts of comparable results were obtained using ACA19D4− transfected and -reverted melanoma cells (data not shown).

These results confirm the conclusion that episialin+ melanoma cells are less susceptible to lysis than episialin− melanoma cells. Importantly, these blocking data also substantiate that episialin does not simply reduce the intrinsic ability of target cells to be lysed.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Relative Fluorescence Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Ag (CLB-LFA-1/1)</td>
</tr>
<tr>
<td>A375</td>
<td>8</td>
</tr>
<tr>
<td>ACA25F2−</td>
<td>7</td>
</tr>
<tr>
<td>ACA25F8+</td>
<td>9</td>
</tr>
<tr>
<td>ACA19D4+</td>
<td>10</td>
</tr>
<tr>
<td>ACA19D4−</td>
<td>9</td>
</tr>
<tr>
<td>ACA30D8+</td>
<td>6</td>
</tr>
<tr>
<td>ACA30D8−</td>
<td>9</td>
</tr>
</tbody>
</table>

Table II

Phenotypical analysis of parental, transfected, and reverted melanoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cytotoxicity (%)a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTL Medium Anti-CD3</td>
</tr>
<tr>
<td>A375</td>
<td>77</td>
</tr>
<tr>
<td>ACA19D4−</td>
<td>45</td>
</tr>
<tr>
<td>ACA19D4+</td>
<td>23</td>
</tr>
<tr>
<td>ACA30D8+</td>
<td>79</td>
</tr>
<tr>
<td>ACA30D8−</td>
<td>59</td>
</tr>
<tr>
<td>518A</td>
<td>12</td>
</tr>
<tr>
<td>MeWo</td>
<td>NT¹</td>
</tr>
<tr>
<td>K562</td>
<td>NT¹</td>
</tr>
</tbody>
</table>

Table III

Cytotoxic activity of CTL and LAK cells against a number of targets cell lines

Effector cells preferentially lyse episialin− target cells

To further investigate whether CTL preferentially lysed the episialin− target cells, we performed cold target inhibition studies. Addition of unlabeled episialin− cells (ACA30D8−) significantly inhibited lysis of both radio-labeled episialin+ and episialin− target cells. Lysis of radio-labeled episialin+ cells (ACA30D8+) was already maximally inhibited after addition of equal numbers of unlabeled episialin− cells, whereas inhibition of lysis of episialin− targets (ACA30D8−) required the addition of significantly larger numbers of unlabeled cells (Fig. 3). Addition of unlabeled episialin+ cells affected lysis of radio-labeled target cells significantly less. An 4- to 8-fold excess of unlabeled ACA30D8+ cells was required to obtain a substantial inhibition of lysis of radio-labeled target cells (Fig. 3). Addition of unlabeled K562 cells did not significantly alter the allospecific lysis of both radio-labeled target cells (Fig. 3). Comparable results were obtained using ACA19D4− transfected and -reverted melanoma cells (data not shown).
Episialin affects adhesion of effector cells to target cells

Next we examined the capacity of the episialin$^+$ target cells (ACA19D4$, ACA30D8$) and episialin$^-$ target cells (ACA19D4$, ACA30D8$) to form conjugates with the various cytotoxic effector cells (LAK cells, CTL). After 2 h of incubation, no effector-target cell conjugates (LAK cells) or only a very low number of conjugates (CTL) were formed with episialin$^+$ cells, whereas 20% and 40% of episialin$^-$ cells formed conjugates with LAK cells and CTL, respectively (Fig. 4A).

Similar results were obtained when CTL were allowed to bind to a monolayer of episialin-transfected melanoma cells or revertants. Figure 4B shows that less CTL bound to episialin$^+$ melanoma cells than to episialin$^-$ cells.

From these results, we conclude that episialin expression on melanoma cells strongly decreases binding to lymphokine-activated killer cells as well as alloantigen activated CTL.

Distinct cell adhesion pathways participate in the cytolytic process

We investigated whether episialin expression affected the contribution of the various adhesion pathways, in particular
LFA-1/ICAM-1 and CD2/LFA-3, in the cytotoxic process. Therefore, we performed mAb inhibition assays. Both lymphokine-stimulated and alloantigen-stimulated cytotoxic activity against episialin+ melanoma cells could be inhibited by addition of mAb directed against LFA-1 and ICAM-1 (60%), whereas lysis of episialin- melanoma cells was much less affected (25% to 15%) (Fig. 5). In contrast, blocking of the CD2/LFA-3 adhesion route resulted in a significant inhibition of specific lysis (CTL) of both episialin+ (45%) and episialin- targets (37%), whereas nonspecific lysis (LAK) was much less affected (Fig. 5). Blocking both adhesion routes (LFA-1/ICAM-1 and CD2/LFA-3) showed additive inhibition (Fig. 5).

From these results, we can conclude that the LFA-1/ICAM adhesion route is mainly involved in lysis of episialin+ melanoma cells, whereas lysis of episialin- cells can be regulated by other adhesion routes. The CD2/LFA-3 interaction plays a substantial role in the lysis of the melanoma cells by alloantigen-activated effector cells, whereas this interaction is not significantly involved in lysis by lymphokine-activated killer cells.

Discussion

We have previously demonstrated that episialin expressed on melanoma cells interferes with cell-cell interaction and prevents homotypic aggregation of these transfected cells (7). The cytolytic process, however, requires a heterotypic attachment of effector cells to target cells, mediated by multiple adhesion receptors and their ligands (32). We hypothesized that episialin expressed by potential target cells (tumor cells) could prevent effector-target cell conjugation and might contribute to the escape of the tumor cells from immune surveillance. The observations that the human mucin DF3 (episialin) is able to inhibit adhesion of eosinophils to antibody-coated targets (33) and that sialomucins, expressed on rat mammary tumor cells, contribute to resistance to natural killer cell-mediated lysis (34), are consistent with this hypothesis. This hypothesis is also supported by a number of observations that emerge from this study: 1) episialin+ melanoma cells are significantly less susceptible to lysis than episialin- melanoma cells by both specific and nonspecific effector cells. This finding is supported by
FIGURE 4. Binding of CTL to episialin-transfected and -reverted melanoma cells. A, The kinetics of heterotypic conjugate formation of effector cells (LAK, CTL) with episialin* target cells ACA19D4* (■), ACA30D8* (○) and episialin* target cells ACA19D4* (□), ACA30D8* (○); and B, binding of CTL to a monolayer of episialin-transfected melanoma cells ACA19D4* and ACA25F8* and episialin-reverted melanoma cells ACA19D4* and ACA25F2*. One representative experiment out of two is shown.

the observation that unlabeled episialin* cells inhibit lysis of labeled target cells more efficiently than unlabeled episialin+ cells. 2) Conjugate formation of both types of cytotoxic effector cells (CTL, LAK cells) with episialin+ target cells can hardly be measured by flow cytometry. Moreover, using another cell adhesion assay (CTL adhesion to monolayers of melanoma cells), which is less sensitive to disruption of CTL-target cell binding, it is demonstrated that CTL adhere significantly less well to episialin+ melanoma cells than to episialin- melanoma cells. Nevertheless, a significant proportion of the episialin+ target cells can be lysed after prolonged times of incubation. These observations may be explained by a brief and unstable contact between effector cells and episialin+ target cells. Not all contact events result in stable adhesion and subsequent lysis of target cells, whereas stable attachment of effector cells to episialin- target cells will result more frequently in target cell lysis. This may also explain the slow kinetics of lysis of episialin+ target cells. 3) The observation that the addition of mAb against LFA-1 and ICAM-1 inhibits lysis of episialin+ cells and not that of episialin- cells by specific and nonspecific effector cells, suggests that this adhesion route, which is involved in the initial contact phase of the cytolytic process (10), is of critical importance in binding effector cells to episialin+ target cells. This observation provides further evidence for the assumption that conjugates of effector cells with episialin+ target cells are unstable and that lysis of these cells requires long incubation periods. Furthermore, it is possible that episialin expression on target cells interfere with effector cell activation. If clustering of receptor molecules is required for this activation, the accessibility of binding sites on the target cells might be disturbed by episialin.

In contrast to the almost complete inhibition of cytotoxic activity of CTL and LAK cells against episialin+ melanoma cells by blocking the two major adhesion pathways (LFA-1/ICAM-1 and CD2/LFA-3), a substantial percentage of episialin- cells is lysed without utilization either one of these two important adhesion pathways (especially by LAK cells). This observation suggests that in the cytolytic pro-
FIGURE 5. Inhibition of adhesion pathways involved in cytotoxic activity of effector cells (LAK and CTL) against episialin+ and episialin− melanoma cell lines. 10 μg/ml purified IgG of each mAb was present in the cytotoxicity assay (4 h). The effector-target cell ratio was 30:1. One representative experiment out of six is shown.

cess against episialin− melanoma cells, a role is reserved for other adhesion pathways besides LFA-1/ICAM-1 and CD2/LFA-3. Melanoma cells express high levels of distinct adhesion molecules of the β1 and β3 group of the integrin family, which are receptor molecules for extra cellular matrix components (35, 36). These molecules may be involved in the cytolitic interaction; however, cellular ligands for most of the β1 and β3 adhesion receptors have not been described. Only the interaction of VLA-4, a β1 integrin, with a cellular ligand, VCAM-1 expressed on endothelial cells, has been documented (37, 38). The clear distinction between episialin− and episialin+ melanoma cells in utilizing additional adhesion molecules may be the result of masking the molecules involved in adhesion routes by episialin. Particularly, the interaction of small transmembrane molecules may be hindered by the long and rigid structure of episialin. This might also explain why some surface molecules on episialin+ cells seem less well expressed compared to those on episialin− cells. Probably, the high episialin expression caused a reduced accessibility for mAb directed against many other surface molecules.

Recently, we have observed that episialin+ melanoma cells, when injected i.v., metastasize in nude mice more efficiently compared to episialin− melanoma cells (J. H. and F. B., unpublished results). This may suggest that episialin+ melanoma cells are less susceptible to the cellular immune response mediated by nonspecific cytotoxic effector cells (NK cells) in nude mice. However, the differences in adhesion properties of the episialin+ and episialin− melanoma cells may not only decrease the sensitivity to the cellular immune response but may also lead to differences in the invasiveness and migration of both cell types.

Finally, it has been reported that despite its antiadhesion function, episialin is still capable of provoking a T cell-mediated immune response, in which an epitope frequently exposed on the repeats of episialin derived by tumor cells is recognized by TCR in a MHC nonrestricted manner (8). Activation of CTL is probably due to TCR cross-linking by specific recognition of repeated epitopes (9). These MHC nonrestricted cytotoxic T lymphocytes were generated from tumor-draining lymph nodes of patients with breast and pancreatic cancers (8, 9). This suggests that the epitope that
is preferentially expressed on malignant cells is capable of inducing an in vivo immune response. However, free episialin sheded by the tumor cells might inhibit the activity of cytotoxic T lymphocytes and thereby protect the tumor cells from this immune recognition.

Acknowledgments

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