Loss of functional E-cadherin renders cells more resistant to the apoptotic agent taxol in vitro

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Abstract

Experimental evidence supports a role for E-cadherin in suppressing invasion, metastasis, and proliferation. Germline mutations of the E-cadherin represent the genetic cause of hereditary diffuse gastric cancer (HDGC). In this type of tumor, isolated cancer cells permeate the basal membrane and paradoxically survive in the gastric wall in the absence of contact with neighbor epithelial cells or with the extracellular matrix. This suggests that upon E-cadherin deregulation, cells acquired resistance to apoptosis. To test this hypothesis, CHO cells stably expressing either wild-type E-cadherin or the HDGC-related germline mutations T340A and V832M were seeded either on a thin layer of collagen type I or on plastic and then subjected to the apoptotic agent taxol. We found that in vitro functional E-cadherin renders cells more sensitive to the effect of taxol. Our results also indicate that this effect is associated to decreased level of the anti-apoptotic bcl-2 protein.

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Introduction

Cell’s survival depends on signals provided by its surroundings. In particular, survival signals can be produced when cells adhere to the extracellular matrix or when intercellular adhesions are formed [1]. In absence of these signals, cells become apoptotic, undergoing a process of anoikis [2,3]. This mechanism is thought to preclude detached epithelial cells (isolated cells) from reattachment and growth outside their home tissue context. Malignant epithelial cells differ from their normal counterpart by becoming resistant to apoptosis upon loss of contact with the basement membrane.

Down-regulation of E-cadherin expression is commonly seen in different tumor types [4–9] and experimental evidence supports its role in suppressing invasion [10]. The possibility that E-cadherin loss could be an early or initiating event in tumorigenesis has come from the study of early hereditary diffuse gastric cancer (HDGC) lesions in germline E-cadherin (CDH1) mutation carriers [6,11]. In this type of cancer, cells carrying an E-cadherin germline mutation invade the surrounding tissues as isolated cells upon loss of intercellular adhesion. There they survive, in
the absence of contact with the extracellular matrix or with neighbor cells [11,12].

We challenged the hypothesis that besides a role in invasion, E-cadherin deregulation could also render the cells more resistant to apoptotic stimuli.

Materials and methods

Cells and culture conditions

Cell lines stably expressing wild-type E-cadherin, as well as its mutants T340A and V832M, were obtained as previously described [13,14]. Mock CHO cells were used as control. Cells were cultured at 37°C under 5% CO2 in humidified air, in α-MEM medium (GIBCO-BRL) supplemented with 5% fetal bovine serum (HYCLONE), 2 mm L-glutamine (GIBCO-BRL), 1% penicillin/streptomycin (GIBCO-BRL), and 500 µg/ml geneticin (GIBCO-BRL) for the selection.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

For the MTT assay, exponentially growing cells (2 x 10^3 per 200 µl per well) were seeded in 96-well plates and were grown for 72 h. Cell survival was evaluated by replacing the culture media by 50 µl of 2.5 mg/ml MTT (Sigma, St Louis, MO, USA), in PBS (pH 7.5). After 4 h of incubation at 37°C in the dark, MTT was removed and 100 µl of Sorenson’s solution (9 mg trisodium citrate, 305 ml distilled water, 195 ml 0.1 N HCl, 500 ml 90% ethanol) was added. The absorbance was determined at 570 nm with a microplate reader (Biorad).

Flow cytometry

For this experiment, cells were plated both on 6-well normal plates and on 6-well plates previously coated with 0.02 mg/cm² collagen type I (BD-Biosciences, Bedford, USA) and cultured for 2 days until they were 60% confluent. Cells were then treated with 0.5 µM taxol for 24 h. Upon treatment, cells were trypsinized and resuspended to produce a single cell suspension, centrifuged at 1500 rpm for 10 min, and fixed with 70% ethanol and recentrifuged. After incubated with RNase (0.5 mg/ml) at 37°C for 30 min, cells were recentrifuged and stained with 1 µg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO), a fluorescent dye that intercalates double-stranded DNA. The DNA profile was then analyzed using a dual laser flow cytometer (Becton Dickinson Flow Cytometer). Cell cycle profiles were analyzed using the ModFIT software.

Table 1

<table>
<thead>
<tr>
<th>Plastic</th>
<th>Taxol, 0 µM</th>
<th>Taxol, 0.5 µM</th>
<th>Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td>7.7 ± 2.35</td>
<td>30.9 ± 2.25</td>
<td>4.43 ± 1.25</td>
</tr>
<tr>
<td>GoG1</td>
<td>52 ± 1.22</td>
<td>11.9 ± 0.96</td>
<td>52.1 ± 1.26</td>
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<tr>
<td>S</td>
<td>6.7 ± 2.25</td>
<td>6.1 ± 1.26</td>
<td>7.58 ± 2.35</td>
</tr>
<tr>
<td>G2-M</td>
<td>33.5 ± 2.11</td>
<td>51.1 ± 1.24</td>
<td>35.9 ± 2.35</td>
</tr>
<tr>
<td>Wild-type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SubG₀</td>
<td>14 ± 5.81</td>
<td>42.3 ± 4.31</td>
<td>6.6 ± 4.71</td>
</tr>
<tr>
<td>GoG1</td>
<td>55.1 ± 4.69</td>
<td>10.3 ± 0.59</td>
<td>51.7 ± 4.00</td>
</tr>
<tr>
<td>S</td>
<td>3.3 ± 5.14</td>
<td>3.2 ± 3.62</td>
<td>6.9 ± 4.85</td>
</tr>
<tr>
<td>G2-M</td>
<td>27.7 ± 5.85</td>
<td>44.2 ± 10.8</td>
<td>34.2 ± 3.86</td>
</tr>
<tr>
<td>T340A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SubG₀</td>
<td>4.3 ± 0.42</td>
<td>29.8 ± 5.86</td>
<td>5.16 ± 0.76</td>
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<tr>
<td>GoG1</td>
<td>57.8 ± 4.32</td>
<td>11.8 ± 3.92</td>
<td>62.6 ± 2.41</td>
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<td>G2-M</td>
<td>31.2 ± 6.21</td>
<td>53.2 ± 2.48</td>
<td>23.7 ± 4.30</td>
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<td>V832M</td>
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<td></td>
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<tr>
<td>SubG₀</td>
<td>13.5 ± 5.49</td>
<td>23.1 ± 4.57</td>
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<td>GoG1</td>
<td>50.1 ± 1.76</td>
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<td>52.3 ± 1.24</td>
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<tr>
<td>S</td>
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<td>11.5 ± 8.80</td>
<td>10.3 ± 0.99</td>
</tr>
<tr>
<td>G2-M</td>
<td>26.8 ± 3.45</td>
<td>53.9 ± 2.10</td>
<td>28.7 ± 0.67</td>
</tr>
</tbody>
</table>

Fig. 1. Percentage of viable cells in the presence of increasing concentration of taxol (0–10 µM). Cells expressing the wild-type E-cadherin showed the lowest D₅₀ dose, indicating increased sensitivity to the apoptotic drug. Differences in D₅₀ between wild-type and each of the other cell lines were statistically significant (t test, P < 0.05). Each data point represents the mean of triplicate analysis.
The TUNEL assay (In Situ Cell Death Detection kit; Roche Molecular Biologicals) was used to detect apoptotic cell death by enzymatic labeling of DNA strand breaks with fluorescein-dUTP and TdT. Briefly, $1 \times 10^5$ cells were seeded either on plastic or a thin layer of collagen type I and incubated or not with 0.5 $\mu$m taxol for 24 h. Alternatively to taxol treatment, cells were grown in serum-free medium for 24 h. Cells were then cytospun onto slides and fixed in 4% formaldehyde/PBS (pH 7.4) for 30 min at room temperature, washed in PBS, and then suspended in permeabilization solution (0.1% Triton X-100/0.1% sodium citrate) for 3 min on ice. Cells were washed again, resuspended in 30 $\mu$l TUNEL-reaction mixture or in 30 $\mu$l label solution alone (negative control), and incubated in a humidified dark chamber at 37°C, followed by washing in PBS. The green fluorescence of apoptotic nuclei was detected by fluorescence microscopy, using a LeicaDM IRE2 Fluorescence Microscope (20× objective). Images were taken with a DC-300F Leica camera and processed with LeicaFW4000 software and Adobe PhotoShop.

**Western blotting and immunohistochemistry**

For Western blot analysis, a total of $5 \times 10^4$ cells were lysed with 50 $\mu$l Triton114 buffer (EDTA 2 mm, PBS Mg$^{2+}$, Ca$^{2+}$ free 1×, Triton-114 1%, DTT 1 mm, Protease inhibitors) and the extracted protein quantified by following the Bradford dye-binding procedure [15]. 10 $\mu$g of protein was separated on a 10% SDS-polyacrylamide gel electrophoresis, followed by transfer onto a...
nitrocellulose membrane. Human bcl-2 mouse monoclonal antibody (Novocastra, NCL-bcl-2-486, 1/500 dilution), rabbit polyclonal antibodies against human cIAP1 and cIAP2 (Santa Cruz Biotechnology, Santa Cruz, CA, 1/1000 dilution), and human AKT mouse monoclonal antibody (Cell Signaling Technology, Beverly, MA 1/1000 dilution), were used for the immunostaining.

For immunohistochemistry, bcl-2 immunostaining was performed with standard immunoperoxidase techniques on 4-μm slides [16], using a bcl-2 monoclonal antibody at a dilution of 1:50.

Results and discussion

To test the hypothesis that E-cadherin deregulation could induce cell resistance to apoptosis, we used CHO cells stably expressing either wild-type or mutated forms of E-cadherin. This cell model was previously used to address the pathogenicity of E-cadherin germline missense mutations in HDGC [13,14]. In particular, for this study, we selected the two germline mutations T340A and V832M, affecting the extracellular domain of the protein and its cytoplasmic tail, respectively. Both mutations were previously shown to confer in vitro protein loss of activity, both in terms of cell-to-cell adhesion and suppression of cell invasion [13,14]. For these cell lines, no differences were previously observed in cell proliferation [17]. Mock E-cadherin negative CHO cells were also used as control. To take into account the effect of cell–matrix interaction in mediating the cellular response to apoptotic stimuli, cells were seeded on plastic or on a thin layer of collagen type I. In all the experiments performed in this study, at least two independent stable clones were used, in order to exclude clonal dependence of the results observed. As previously shown [13,14,17], all cell lines but mock CHO cells expressed comparable amount of transfected E-cadherin.

We assessed the effect of the apoptotic agent taxol (paclitaxel) on cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The metabolic activity of viable cells was estimated colorimetrically, as a function of their ability to convert MTT in a colored formazan salt in the presence of increasing concentrations of taxol (Fig. 1). Cells expressing wild-type E-cadherin resulted the most sensitive to the

![Fig. 4](image-url)

Fig. 4. (a) DAPI staining of nuclei of cells grown in the absence (–taxol) or upon incubation with 0.5 μM taxol (+taxol). Nuclear condensation as well as apoptotic bodies’ formation are markedly visible in cells expressing wild-type E-cadherin upon taxol treatment. Similar results were obtained upon serum withdrawal, independently of the presence of the collagen matrix. (b) Percentage of apoptotic cells as estimated by TUNEL assay. Cells were grown either in the presence of serum (+serum) or under serum starvation conditions (–serum). Bars represent the percentage of fluorescent cells, as a function of the total number of cells. Cells were counted in at least three different fields. Only results obtained for cells seeded on collagen type I are shown, since no differences were observed when compared to cells seeded on plastic (t test, P > 0.05). Upon serum withdrawal, we observed a significant increase in the percentage of apoptotic cells only for cells stably expressing wild-type E-cadherin (t test, P < 0.05).
apoptotic agent (D$_{50}$: 2.1 ± 0.27 μM), in comparison to both mock cells (D$_{50}$: 3.7 ± 0.32 μM) and cells expressing the E-cadherin missense mutations T340A (D$_{50}$: 4.0 ± 0.16 μM) and V832M (D$_{50}$: 7.8 ± 1.4 μM). On the basis of this result, we estimated that 0.5 μM taxol (the lowest dose at which differences between cell lines are already observed) would be the most suitable concentration of drug to be used in the experiments that followed. We determined by flow cytometry the percentage of apoptotic cells in the presence of 0.5 μM of paclitaxel, after seeding the cells either on plastic substrate (P) or collagen type I (C). Taxol is known to stabilize tubulin dimers, thus interfering with microtubular disassembly and resulting in the arrest of cells in G2-M phase [18]. In keeping with this, all cell lines tested showed an increase in the population of cells arrested in the G2-M phase upon incubation with the drug (Table 1). The percentage of cells in SubG$_0$ phase with respect to the presence of taxol and/or collagen type I is shown in Fig. 2 and Table 1. In the absence of apoptotic stimuli, all cell lines showed comparable levels of apoptotic cells on plastic or collagen. Incubation with taxol induced apoptosis in all cell lines, but the extent of its effect was significantly more pronounced for cells expressing wild-type E-cadherin. Differences in the response of cells to the apoptotic agent were however independent on the presence of collagen type I, supporting the idea that in our in vitro model, the mechanism underlying the resistance to the apoptotic agent upon loss of functional E-cadherin is dependent on the E-cadherin status itself. These results were confirmed by TUNEL assay, by estimating DNA strand breaks in fluorescent individual cells, upon incubation with 0.5 μM of paclitaxel (Fig. 3). In response to the apoptotic stimulus, wild-type E-cadherin expressing cells showed three to four times as much positive apoptotic cells as the other tested cell lines, independently of the presence of the collagen matrix. In keeping, marked nuclear condensation and formation of apoptotic bodies were visible on DAPI-labeled nuclei of cells expressing wild-type E-cadherin (Fig. 4a). As alternative to taxol treatment, we also used serum starvation as a more physiological inducer of apoptosis. According to what was observed upon taxol treatment, a threefold increase in apoptotic cells was observed for wild-type expressing cells (Fig. 4b).

The finding that cells lacking functional E-cadherin appear less sensitive to taxol, would also fit with the observation that in HDGC E-cadherin acts as a classical tumor suppressor gene, leading to tumor development only upon loss of the wild-type allele. It is therefore predictable that resistance to apoptosis due to E-cadherin deregulation may not happen in the presence of a wild-type allele.

Because commitment to apoptosis is usually defined by the relative expression of pro- and anti-apoptotic proteins, E-cadherin deregulation might interfere with this balance, thus leading to an increased resistance of the cell to apoptosis. In this regard, Sasaki et al. [19] reported that in rat breast carcinoma cells, overexpression of wild-type E-cadherin reduced the expression of bcl-2, rendering the cells more sensitivity to etoposide-induced apoptosis. These findings, together with the observation that paclitaxel, besides its effect on mitotic spindle formation during mitosis, is also able to bind and phosphorylate the bcl-2 protein causing its inactivation [20], led us to hypothesize that the increased resistance to apoptosis observed for cells expressing non-functional E-cadherin could be transmitted through the anti-apoptotic bcl-2.

We characterized the level of bcl-2 protein by Western blot analysis in the four cell lines (Fig. 5a). Cell lines expressing the mutants T340A and V832M as well as mock cells showed higher levels of bcl-2 protein than cells expressing wild-type E-cadherin, suggesting a possible
correlation between E-cadherin and bcl-2 regulation. To support this observation, we characterized by Western blotting the expression level of cIAP-1 and cIAP-2 (two known anti-apoptotic proteins of the IPA's family) as well as AKT (a key mediator of different signaling pathways) (Fig. 5a). All cell lines expressed comparable amount of the three proteins, strengthening the finding of decreased level of bcl-2 protein in cells expressing functional E-cadherin.

Since we had access to the primary tumors of carriers of the V832M and T340A E-cadherin germline missense mutations, we characterized the expression of bcl-2 by immunohistochemistry. As shown in Fig. 5b for the V832M mutation, invasive isolated signet ring carcinoma cells showed positive bcl-2 cytoplasmic staining, thus supporting the in vitro observation. In the case of the T340A mutation, we failed to obtain any result, likely because the material available was too old and not properly preserved. Previously, Fricke et al. [21] reported a series of 24 sporadic diffuse gastric cancers, 16 of which were positive for E-cadherin mutation. Bcl-2 overexpression was observed in 5 of the E-cadherin mutation positive cases but in none of the 8 remaining cases. This could indicate that E-cadherin deregulation is a necessary but not sufficient condition for bcl-2 up-regulation, whereas the specific cellular environment is likely to play a major role in mediating this interplay.

In conclusion, we showed that in our in vitro model, upon transfection of wild-type E-cadherin, cells become more sensitive to apoptotic stimuli, a reversion of phenotype that is not observed when mutated E-cadherin is transfected. This could be of relevance for the understanding of the tumorigenic process dependent of E-cadherin deregulation. We also observed a decrease in the level of the anti-apoptotic bcl-2 in cells expressing wild-type E-cadherin, suggesting but not proving the existence of an interplay between E-cadherin and bcl-2 regulation. Because of the potential therapeutic relevance of this finding, further studies aiming at elucidating its molecular mechanisms are warranted.

Acknowledgments


References