E-cadherin decreased human breast cancer cells sensitivity to staurosporine by up-regulating Bcl-2 expression *

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A B S T R A C T

E-cadherin, a well-characterized cell–cell adhesion molecule, executes multifunction roles on cell behaviors. However, its effect on chemo-resistance remains controversial. In this study, we found that E-cadherin positive breast cell lines were less sensitive to staurosporine compared to E-cadherin negative ones. Next, we substantiated that the expression of E-cadherin in MDA-MB-435 cells could partly counteract the cytotoxic effect induced by staurosporine through a series of apoptosis assay. The resistance of E-cadherin over-expressing cells to staurosporine may due to the up-regulation of Bcl-2/Bax ratio. When E-cadherin interference plasmids were transfected into MCF-7 cells, Bcl-2 expression was down-regulated. Moreover, perturbation of E-cadherin function by blocking the cell–cell contact resulted in decreased cellular levels of Bcl-2 protein expression. All these results demonstrated the chemo-resistance function of E-cadherin in the condition of staurosporine treatment, therefore, might contribute effective therapeutic strategies in breast carcinoma.

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Introduction

Apopotosis, a type of programmed cell death, is an evolutionarily conserved biochemical pathway in controlling cell suicide that plays an essential role in regulating normal development and homoeostasis in multicellular organisms [1]. Induction of apoptosis is considered to be the underlying mechanism that accounts for the efficiency of most chemotherapy drugs. Staurosporine (STS) 2, a potent protein kinase C inhibitor with a broad spectrum of activity, is used in vitro as an initiator of apoptosis in a wide variety of cell types. Nevertheless, STS has not been used as a clinical chemotherapy drugs until now. The potential factors that influence the STS-induced apoptosis remain largely unknown.

Generally, it is believed that a mitochondrial pathway plays a critical role in STS-induced apoptosis [2]. This involves release of mitochondrial apoptotic proteins such as cytochrome c, apoptosis-inducing factor (AIF) and second mitochondrial-derived activator of caspase (SMAC) [3–5]. On release, cytochrome c interacts with apoptotic proteinase-activating factor-1 and pro-caspase-9 to form apoptosomes. The latter activates caspase-9 and downstream effector caspases such as caspase-3 that are responsible for apoptotic destruction of the cells [6]. The anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-XL and MCL1 appear to preserve the integrity of outer mitochondrial membrane [7,8]. Over-expressions of Bcl-2 and Bcl-XL inhibit mitochondria-dependent pathway to apoptosis in different kinds of cells, thus protecting those cells from killing by chemotherapeutic agents [9,10]. And reduction of Bcl-2 levels increases the activity of chemotherapy against many tumor types in vitro and in vivo [10–14]. As such, the proto-oncogene Bcl-2 is thought to be directly associated with cellular transformation and resistance chemotherapy [9,15].

E-cadherin, a well-characterized cell adhesion molecule, is essential for cell–cell adhesion, which in turn regulates various aspects of cell fate including developmental decisions, cellular differentiation and possibly cell survival [16,17]. Just as integrins’ function to mediate cell–extracellular matrix interactions in anchorage-dependent survival, cadherins may also act in such a capacity, possessing a functional role in the regulation of intercellular adhesion-dependent survival. Several studies have reported the association between E-cadherin-mediated aggregation and survival of carcinoma cells [18]. However, the actual mechanism by which cadherins mediate these signals is not known. As a hallmark of tumor progression, E-cadherin expression varies in a larger range in different types and stages of tumor [19,20]. It would be much helpful for the applications
of chemotherapy if the relationship between the E-cadherin expression level and the susceptibility of tumor cells to chemotherapy drugs be clarified.

We investigated in this report that E-cadherin over-expressing breast cancer cells were less sensitive to STS, thereby reducing the effectiveness of chemotherapy. The mechanism by which E-cadherin protected cells from STS-induced apoptosis was due to the up-regulation of Bcl-2.

Materials and methods

Cell culture

The human breast carcinoma cell lines MDA-MB-435, MDA-MB-231, T47D and MCF-7 were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% new-born bovine serum at 37 °C, at 5% CO₂.

MTT cell viability assay

Cell proliferation and viability were quantified using the methyl thiazol tetrazolium (MTT) cell proliferation reagent (Roche Diagnostics) according to the manufacturer’s protocol. Cells were seeded at a density of 2×10⁵ cells/ml into 96-well plates and incubated for 12 or 24h in completed medium with DMSO (control) or supplemented with various dose of STS; then 20μl MTT labeling solution (5mg/ml) per well was added. After incubation at 37°C for 4h, culture medium was discarded and 150μl DMSO was added to each well. The samples were quantified spectrophotometrically by measuring the absorbance of the formazan product at 490 nm with an ELISA plate reader.

Stable transfection

The MDA-MB-435 cells (1×10⁶/well) were transfected with pcDNA3-E-cadherin or pcDNA3 empty vector plasmids (4μg/well) in 6-well plates using Lipofectamine™ 2000 (Invitrogen Inc., Carlsbad, CA), according to manufacturer’s guidelines. Twenty-four hours after transfection, the cells were seeded into 100nm dishes with a dilution of 1:20 and cultured in DMEM with 800μg/ml of G418 (Promega, Pittsburgh, PA) for selection. The anti-G41 clones were further screened by Western blot (see [21]), and then one of the E-cadherin expressing stable clones named Ecad6-435 and an empty vector pcDNA3 stable clone named Neo1-435 were selected for next investigation. The stable clones, which were maintained in DMEM with 200μg/ml of G418, were validated for their E-cadherin expression every three month.

Immunofluorescent staining

The human breast carcinoma cell lines MDA-MB-435 cells were plated at densities of 2×10⁴ cells/cm² onto glass coverslips and grown for overnight. Cells were washed twice in 1× PBS, and fixed in 4% (v/v) paraformaldehyde for 10min at room temperature. Then the fixed cells were incubated with primary antibody against E-cadherin (diluted 1:500) for 6h at 37°C, washed three times with 1× PBS followed by treatment with FITC-conjugated secondary antibody (diluted 1:200) for 1h at 37°C. Negative control was produced as mentioned above except for omitting the primary antibody. The immunofluorescently stained cells were examined on the OLYMPUS™ microscope.

Cell aggregation assay

Cells were detached with HCMF buffer (150 mM NaCl, 0.6 mM Na₂HPO₄, 10 mM glucose and 10 mM Hepes, pH 7.4) containing 0.02% trypsin and 2 mM CaCl₂. Single cell suspensions were re-suspended in HCMF buffer containing 2 mM CaCl₂ at a concentration of 10⁶ cells/ml. Aliquots of cell suspension (300 ml) were added to each well of 24-well plates pre-coated with 1% BSA and incubated for different times at 37°C with 80rpm agitation. The aggregation index was calculated by (N₀ - Nₜ)/N₀, where N₀ was the number of particles before the experiment started, and Nₜ was the number at final time.

DAPI staining

To visualize DNA, cells were washed twice with 1×PBS, fixed for 30min in 4% paraformaldehyde, incubated with 4’,6’-diamidine-2-phenyldine hydrochloride (DAPI) for 10min, and then analyzed under a fluorescence microscope (OLYMPSUS).

Annexin V apoptosis assay

For apoptosis quantification by annexin V, cells were scraped and stained with annexin V-FITC and propidium iodide according to the manufacturer’s instructions (Annexin V-FITC Apoptosis Detection Kit, Beyotime institute of biotechnology, China). In brief, cells after treatment with STS were washed with PBS/BSA. After the addition of 195μl binding buffer, 5μl FITC-labeled annexin V were added and incubated for 10min at room temperature. Followed by incubation with 10μl propidium iodide for 10min on ice in the dark, apoptotic cells were measured by FACs analysis.

Immunoblotting assay

Immunoblotting analysis was carried out as described previously [21]. In brief, the separated proteins were transferred onto a PVDF membrane and probed with the appropriate primary antibodies and horseradish peroxidase labeled-secondary antibodies, and then detected using the ECL kit (Pierce).

E-cadherin SiRNA plasmid construct

SiRNA specific for E-cadherin (NM_004360) was designed according to a reported DNA sequence of the type AA(N19) (CCAGAGTTTGCTTCATGTTAC). According to Ambion company’s guideline, a pair of oligonucleotides (sense: 5’-GATCCGCGAATTTGCTCAATTTCTCTACTATCGAGAGATACCTGCAACCTCTGTTTTTGGAAA-3’ antisense: 5’-AGCTTGCATACCGAGTCTGCTTCTTGTCTCTTGCTAGATTTGAGAGATACCTGCAACCTCTGTTTTTGGAAA-3’) was synthesized, annealed and ligated to the BamHI and HindIII sites of pSilencer 2.0 (Ambion Co., USA) to get plasmid pSilencer 2.0-E-cadherin. The inserts were confirmed by sequencing. The negative control vector (control) was purchased from Ambion.

E-cadherin functional blocking experiments

Incubation was conducted with either monoclonal anti-E-cadherin antibody DECMA-1 (Sigma, U3254) or calcium chelator EGTA as described previously [21]. In brief, 0, 5, 10 and 20 μg/ml DECMA-1 or 0, 0.01, 0.1, 0.5, 1 and 2 mM EGTA was added into the ~95% confluent Ecad6-435 cells, separately, and incubated for 12h. Then these treated cells were collected and lysed for Western blot analysis.

Statistical analysis

The Western blotting experiment was performed at least three times. For the MTT cell proliferation assay, three experiments with seven parallels (n=21) were tested for every concentration.
repeated three times * 

The aggregation index was calculated by (C) Cell aggregation analysis of breast cancer cell line.

Fig. 2. Characterization of positive expression of E-cadherin transfectants in breast cancer cells MDA-MB-435 

To study the effect of STS on human breast cancer cells, E-cadherin negative cell lines (MDA-MB-435 and MDA-MB-231) and E-cadherin positive cell lines (MCF-7 and T47D) were treated with various doses of STS for 24 h, and assayed for growth inhibition. As shown in Fig. 1, STS treatment resulted in a loss of cell viability in a dose-dependent manner in each cell line. Among four breast carcinoma cell lines, MDA-MB-435 was the most sensitive cell line towards STS with IC50 value of 0.56 μmol/L, whereas MCF-7 was the least sensitive cell line with IC50 value of 8.3 μmol/L. The cytotoxicity of STS to E-cadherin null cells were markedly greater (IC50 of 0.56 and 0.69 μmol/L) compared with that of E-cadherin positive cells (IC50 8.3 and 2.0 μmol/L). Thus, it seemed that the expression of E-cadherin counteracted the cytotoxic effects induced by STS in breast cancer cells.

Results

Effect of STS on the growth inhibition of E-cadherin negative and positive breast cancer cells

To determine whether E-cadherin regulated STS-induced breast cancer cells apoptosis, MDA-MB-435 cells (E-cadherin null) were transfected with pcDNA3-E-cadherin expression plasmid or pcDNA3 empty vector to build their stable transfectants, which were designated as Ecad6-435 and Neo1-435, respectively. Then, the E-cadherin expression of every transfectant was validated. As shown in Fig. 2A, E-cadherin protein level was significantly increased in Ecad6-435 cells, whereas hardly detected in Neo1-435 cells. Next, we tested the function of the exogenous E-cadherin in Ecad6-435 cells. Using immunofluorescent staining, we found that exogenous E-cadherin could locate to the membrane surface in Ecad6-435 cells, suggesting the build-up of E-cadherin-mediated cell–cell adhesion (Fig. 2B). Cell aggregation assay, which further confirmed this assume, showed that positive expression of E-cadherin significantly increased the cell–cell adhesion in Ecad6-435 cells. (Fig. 2C) These results demonstrated that the E-cadherin-mediated cell–cell adhesion had been built up in Ecad6-435 cells.

E-cadherin expression repressed STS-induced apoptosis

To test the effect of E-cadherin on protecting breast carcinoma cells from STS-induced apoptosis, STS stimulation of Ecad6-435 and Neo1-435 cells was conducted and the percentage of viable cells was determined by the MTT assay, firstly. At each concentration level of STS, Neo1-435 cells were induced to death with...
A larger proportion than that of Ecad6-435 cells. The extreme difference of cell viability between Neo1-435 and Ecad6-435 cells occurred at a 2 μM STS treatment, where cell viability in Ecad6-435 cells was about 55% of its untreated control level in comparison to that in Neo1-435 cells which was only 23% of its control level (Fig. 3A). Then, we examined the apoptosis by testing DNA fragmentation and poly(ADP-ribose) polymerase (PARP) cleavage. As shown in Fig. 3B, treatment of Neo1-435 cells with STS resulted in PARP significant cleavage, whereas Ecad6-435 cells had minimal amount of cleaved PARP. We also found that DNA fragmentation was more easily induced in Neo1-435 than in Ecad6-435 cells upon a 5 μM STS stimulation using DAPI staining (Fig. 3C).

One of the membrane changes in apoptosis or necrosis is the translocation of phosphatidylserine (PS) from the inner of the cell membrane to the outside. To further evaluate the efficiency of STS-induced apoptosis in those stable clones, Annexin V/PI staining assay was carried. In Neo1-435 cells, within 5 h after various doses of STS treatment, annexin V labeling was significantly increased from approximately 4% to 24% (Fig. 3D, upper), suggesting a rapid induction of apoptosis. Whereas, in Ecad6-435 cells, there appeared to be a slight increase in annexin V staining which was from approximately 4% to 9% (Fig. 3D, lower). This further substantiated E-cadherin expression might impair STS-induced apoptosis in MDA-MB-435 cells.

E-cadherin expression up-regulated Bcl-2 protein level

Given that the interplay between prosurvival and pro-apoptotic Bcl-2 family proteins is capable of controlling anticancer drug-induced apoptosis, we examined the expression of Bcl-2 and Bax in Ecad6-435 and Neo1-435 cells. Using quantitative Western blot-
Discus sion

After E-cad herin-med i ated adhe sion was used. After tran siently trans fec ted with MCF-7 cells in a dose-depen dent manner (Fig. 4B), the data sug gested the Bcl-2 protein might be a de ter minant fac tor that reg u lated the quan ti fi ca tion of Bcl-2 protein expres sion. Inhi bi tion of functional E-cad herin, there fore, might con- tribute effec tive ther a peu tic strat e gies in breast carci noma.

Dis ruption of hom ophil ic bind ing of the E-cad herin ex tra cel lu lar do main by DECMA-1 or EGTA cor re lated with decreased cel lular lev els of Bcl-2 pro tein expres sion

Since the cal cium-depen dent inter  ac tions among E-cad herin mole cules are cri tical for the for ma tion and main ten ance of cell–cell adhe sion, we uti lized cal cium che la tor EGTA and a neu tral iz- ing anti body against E-cad herin (Fig. 4A). To fur ther val i date the ini tia tion of cell death pro grams as well. The effect of E-cad herin up-reg u lated cell–cell adhe sion on cell sur vival and apop to sis has been re ported to pre vents cells from apop to sis in dif fer ent kinds of cells, which di rectly or indi rectly antag o niz ing the tumors...  These ob serva tions sug gested that the main te nance of E-cad herin-med i ated cell–cell adhe sion was nec es sary for the up-reg u la tion of cell-cell adhe sion led to a decrease of pro-apop to tic Bcl-2 expres sion. This work con firmed the chemo-resis ten tance func tion of E-cad herin, there fore, might con- tribute effec tive ther a peu tic strat e gies in breast carci noma.

The sig nal ing induced by E-cad herin-med i ated cell–cell adhe sion is com plex. We here showed that E-cad herin up-reg ulated Bcl-2 expres sion. E-cad herin engagement in duces several sig nal ing pathways in clud ing β-catenin/wnt, Akt and EGFR sig nal ing [27–30]. Future stud ies will be needed to elu ci date the com plicated cross talk
of cell surface adhesion molecules with apoptosis-related signaling pathways. Studies have shown that ERK/MAPK can increase the level of Bcl-2 through the phosphorylation of Bad in SCC cells [31]. On the premise of this, an appealing hypothesis was to test the possibility that E-cadherin engagement is the trigger for anti-apoptotic signaling via ERK/MAPK signaling.

In conclusion, we showed that in this in vitro model, upon transfection of wild-type E-cadherin, cells become less sensitive to apoptotic stimuli. This could be of relevance for the understanding of the tumorigenic process in the E-cadherin wild-type breast cells. We also observed an increase in the level of the anti-apoptotic Bcl-2 in E-cadherin over-expressing cells, suggesting 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.

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References