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Effects of Cd^{2+} on *cis*-dimer structure of E-cadherin in living cells

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ABSTRACT

E-cadherin, a calcium (Ca^{2+})-dependent cell–cell adhesion molecule, plays a key role in the maintenance of tissue integrity. We have previously demonstrated that E-cadherin functions *in vivo* as a *cis*-dimer through chemical cross-linking reagents. Ca^{2+} plays an important role in the *cis*-dimer formation of cadherin. However, the molecular mechanisms by which Ca^{2+} interacts with the binding sites that regulate *cis*-dimer structures have not been completely elucidated.

As expected for a Ca^{2+} antagonist, cadmium (Cd^{2+}) disrupts cadherin function by displacing Ca^{2+} from its binding sites on the cadherin molecules. We used Cd^{2+} as a probe for investigating the role of Ca^{2+} in the dynamics of the E-cadherin extracellular region that involve *cis*-dimer formation and adhesion. While cell–cell adhesion assembly was completely disrupted in the presence of Cd^{2+}, the amount of *cis*-dimers of E-cadherin that formed at the cell surface was not affected. In our "Cd^{2+}-switch" experiments, we did not find that Cd^{2+}-induced E-cadherin *cis*-dimer formation in EL cells when they were incubated in low-Ca^{2+} medium.

In the present study, we demonstrated for the first time the effects of Cd^{2+} on the *cis*-dimer structure of E-cadherin in living cells using a chemical cross-link analysis.

1. Introduction

Cadherin-based cell–cell adhesion is essential for the morphogenesis of tissues and the maintenance of tissue function [1,2]. The three-dimensional structure of the extracellular domain bearing the adhesion site has been resolved for N-cadherin [3], E-cadherin [4], and C-cadherin [5]; the structures that have been described to date suggest that the extracellular domains of cadherins exist as stable, *cis*-dimers that are necessary for intrinsic homophilic cell–cell adhesion activity. Various experimental results have suggested that lateral interactions may be an important feature of cadherin adhesive functions [6,7].

The extracellular region of cadherin, which plays an important role in cadherin-mediated cell adhesion, has five tandemly repeated ectodomains (EC1–EC5) with three calcium (Ca^{2+})-binding sites that are situated between each of these domains. Structural studies have suggested that the binding of Ca^{2+} induces a conformational change in EC1 by undocking the conserved tryptophan-2 from its own hydrophobic pocket and docking it into the hydrophobic pocket of its adhesive partner, which leads to cell–cell adhesion [5,8]. Therefore, cadherins depend on Ca^{2+} for their function, and the removal of Ca^{2+} abolishes the adhesive activity [9]. Cadherin loses its rigidity and becomes a very flexible structure at low Ca^{2+} concentrations, and Ca^{2+} is believed to rigidify the extracellular portion of the protein, which, when complexed, adopts a rod-like conformation [7,9,10]. The Ca^{2+}-dependent rigidity of the interdomain regions of classical cadherins has been shown by NMR analyses of Ca^{2+}-free E-cadherin [11], and electron microscopic images have also shown the collapse of rigid E-cadherin ectodomains upon Ca^{2+} removal [12]. Despite these results, the relative importance of the different Ca^{2+}-binding sites at the cadherin extracellular domain interfaces remains unclear.

The role of Ca^{2+} in the *cis*-dimer formation of cadherin has been suggested by the results of previous studies [5,13]. Our previous study has also indicated that the *cis*-dimer formation of E-cadherin *in vivo* is dependent on the presence of Ca^{2+} [14]. However, the molecular mechanisms by which Ca^{2+} interacts with the binding sites that regulate *cis*-dimer structures have not been completely elucidated.

Cadmium (Cd^{2+}) is an important environmental pollutant that has been shown to cause severe damage to various organ systems. As expected for a Ca^{2+} antagonist, Cd^{2+} disrupts cadherin function by displacing Ca^{2+} from its binding sites on the cadherin molecules [15,16]. Therefore, we have used Cd^{2+} as a probe for investigating the role of Ca^{2+} in the dynamics of the E-cadherin extracellular region that involves *cis*-dimer formation and cell adhesion.

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The objective of the present study was to examine the effects of Cd^{2+} on the dynamics that regulate the *cis*-dimer formation of E-cadherin *in vivo*.

2. Materials and methods

2.1. Cell culture

EL cells [14] (mouse fibroblast L cells expressing mouse E-cadherin) were maintained in DMEM supplemented with 10% fetal bovine serum.

2.2. Reagents and antibodies

Antibodies specific for E-cadherin and, purchased from BD Biosciences (San Jose, CA, USA) were used for immunoblotting. A monoclonal antibody specific for E-cadherin (DECMA-1; Sigma–Aldrich Co. LLC, St. Louis, MO, USA) was used for immunocytochemistry. Cadmium chloride ($CdCl_2$) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.3. Immunocytochemistry

EL cells were grown on poly-L-lysine-coated glass coverslips and immunofluorescence was performed as previously described [17].

2.4. Cell aggregation assay

The cell aggregation assay was performed as described previously [14].

2.5. Determination of LDH release

Lactate dehydrogenase (LDH) release corresponds to the integrity of the cell membrane [18]. LDH release into medium was measured with a CytoTox96 Non-Radioactive Cytotoxicity Assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions.

2.6. Chemical cross-linking and immunoblotting

We used the cell membrane-impermeable chemical cross-linker bis sulfosuccinimidyl suberate (BS^3) (Thermo Fisher Scientific Inc., Rockford, IL, USA) to label the cell surface. Another type of chemical cross-linker, 1,8-bis-maleimidotriethyleneglycol ($BM[PEO]^3$) (Thermo Fisher Scientific Inc.), was also used for labeling the EL cell surface. After treatment with the cross-linker, the EL cells were solubilized. Immunoblot was analyzed as previously described [14].

2.7. Cell surface biotinylation

Sulfo-NHS-Biotin (Thermo Fisher Scientific Inc.) was used for EL cell surface biotinylation. The biotinylation procedure was performed according to the manufacturer's instructions. The biotinylated proteins were affinity purified by streptavidin–agarose (Thermo Fisher Scientific Inc.) and subjected to immunoblotting.

2.8. Calcium and cadmium switch assay

For the calcium and cadmium switch experiments, confluent EL cells were incubated overnight in a low-Ca^{2+} medium [19]. The medium was then switched to a medium containing Ca^{2+} or Cd^{2+} for 4 h.

3. Results

3.1. Influence of cadmium on EL cell morphology, E-cadherin localization, and cell adhesive activity

EL cells showed the cobblestone appearance of a normal epithelial-like monolayer (Fig. 1A, 0 h). As shown in Fig. 1A, when EL cells were treated with 100 μM $CdCl_2$, they became rounded and lost almost all cell–cell contacts. These results were consistent with those of previous studies [20,21]. These effects resembled those that occurred when the cells were incubated in the presence of a Ca^{2+} chelator [12].

In order to examine the pattern of localization of E-cadherin in $CdCl_2$-treated EL cells, an immunofluorescence analysis was performed (Fig. 1B). As shown in Fig. 1B, EL cells demonstrated the typical pattern of E-cadherin expression at the cell borders under control conditions (0 h) and with a 2 h incubation (2 h). After a 4 h exposure to 100 μM $CdCl_2$, clusters of EL cells showed a clearly damaged E-cadherin pattern with a morphological cellular change (Fig. 1B, 4 and, 6 h).

Interestingly, the cell–cell contact was no longer recognizable in substantial parts of the EL cells, but E-cadherin protein could still be detected as intensely stained spots (arrows in Fig. 1B, 6 h) in the cell periphery after 4 h of incubation. In order to examine the localization of the intense E-cadherin-stained spot, an immunofluorescence analysis was performed on non-permeabilized EL cells (Fig. 1D). An antibody that was specific for the extracellular domain of E-cadherin (DECMA-1) was also positive in the non-permeabilized EL cells that showed the intensely stained E-cadherin spots on their cell surface (Fig. 1D, 6 h). Our observations indicated that E-cadherin coalesced into punctate aggregates on the cell surface following Cd^{2+} treatment.

The effects of Cd^{2+} on cadherin-mediated cell adhesive activity were examined by a cell aggregation assay [11]. The aggregates of EL cells in the absence of Cd^{2+} showed extensive cadherin-mediated "compaction" (Fig. 1C, 0 h), which is when cells adhere tightly to each other and, change in shape to maximize the contact areas [22,23]. However, the aggregates of EL cells that were in the presence of Cd^{2+} did not show any appreciable morphological changes, and each cell in the aggregate remained round and was easily distinguishable (Fig. 1C, 2 and 4 h). In addition, no cell aggregation was observed in the EL cells with 6 of h incubation of 100 μM $CdCl_2$ (Fig. 1A). Under these conditions, a "de-compaction" effect of Cd^{2+} was apparent in the presence of physiological concentration of Ca^{2+}.

Taken together, the cadherin-mediated cell adhesive activities of EL cells were markedly reduced in a time-dependent manner during the 6 h of incubation, as shown in Fig. 1C (0, 2, 4, and 6 h).

3.2. Cadmium cytotoxicity

LDH leakage is a sensitive indicator of cytotoxicity [18]. The cadmium cytotoxicity was examined by LDH release in the medium. In these experimental conditions, no significant changes in the LDH concentrations in the medium were observed in the EL cells following incubation with 100 μM $CdCl_2$ for 0, 2, 4 and 6 h (Table 1). These results indicated that EL cell membrane integrity was not affected by treatments that lasted for at least 6 h.

3.3. The effects of cadmium on the cis-dimer structure of E-cadherin at the EL cell surface

We next examined the $CdCl_2$ effects on the *cis*-dimer form of E-cadherin at the EL cell surface with a chemical cross-linking analysis. Our previous studies have indicated that the chemical

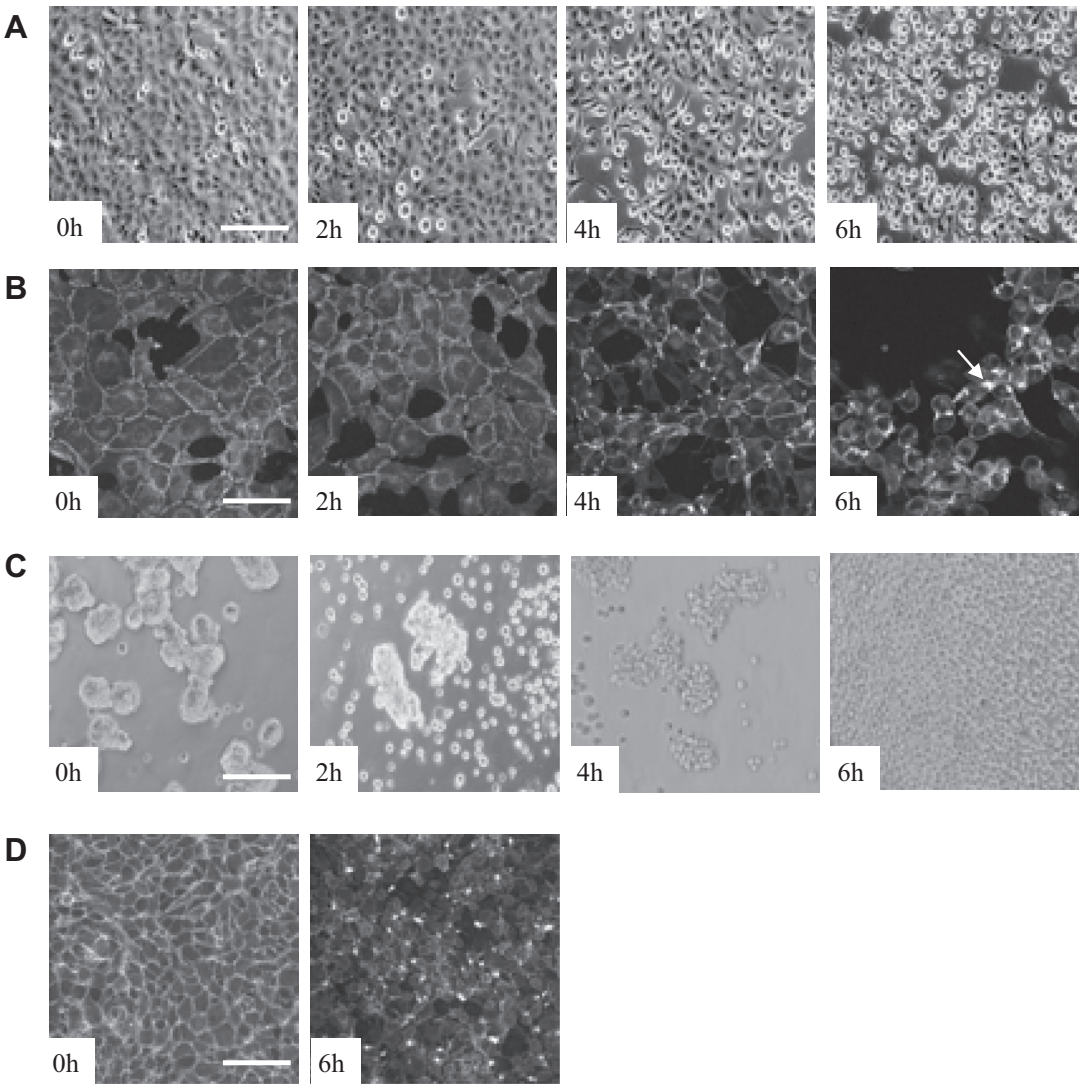

Fig. 1. Influence of $CdCl_2$ on EL cell morphology and cadherin-based cell–cell adhesive activity. (A) The phenotypic change in EL cells after treatment with 100 mM of $CdCl_2$. Bar = 20 μm. (B) Immunofluorescence microscopy of the E-cadherin pattern. Bar = 20 μm. (C) Cell aggregation assay Bar = 50 μm. (D) Immunofluorescence microscopy of the E-cadherin pattern in non-permeabilized EL cells. Bar = 20 μm.

Table 1
Cell cytotoxicity (%) of EL cells after the addition of 100 μM $CdCl_2$.

Incubation time (h)	Cell cytotoxicity (%)
0	0.3782 + 0.0084
2	0.3806 + 0.0068
4	0.3844 + 0.0089
6	0.3888 + 0.0096

Mean + S.D; $n = 9$.
EL cells were exposed to 100 μM $CdCl_2$. After exposure, the supernatants were collected and evaluated for cytotoxicity [lactate dehydrogenase (LDH) release]. The activity of LDH that was released into the culture medium was measured. Cell cytotoxicity (%) was calculated according to the manufacturer's instructions. The results are represented as a percentage of the total LDH activity.

cross-linker BS^3 could capture the *cis*-dimer form, but not the *trans*-dimer form of mouse E-cadherin on the cell surface [14].

Fig. 2A indicates that the total amount of E-cadherin that was expressed in the EL cells was not affected by 100 μM $CdCl_2$ treatment for 6 h. No significant change in the amount of cross-linked E-cadherin on the EL cell surface was observed during 6 h of incubation of 100 μM $CdCl_2$ as shown in Fig. 2A (lanes 6–10).

We next examined the amount of chemical cross-linking of E-cadherin with another type of chemical cross-linker, $BM[PEO]^3$ (Fig. 2B). $BM[PEO]^3$, which is a homo-bifunctional cross-linker for conjugation between sulfhydryl groups (–SH), was applied in order to study cadherin dimerization on the surface of epithelial A-431 cells [24]. E-cadherin contains five cysteine residues. Four of them, which were present at the EC5 domain, form two disulfide bonds [25], and the potential stabilizing role of a disulfide-bond between the sulfhydryl groups in the monomers forming an E-cadherin *cis*-dimer has also been reported [12]. As shown in Fig. 2B, the amount of E-cadherin (*cis*-dimer form) that was cross-linked with $BM[PEO]^3$ was not affected in the absence and presence of 100 μM $CdCl_2$.

A cell surface biotinylation assay was performed in order to determine the amount of cell surface E-cadherin during $CdCl_2$ treatment. Fig. 2C shows that 6 h of 100 μM $CdCl_2$ incubation did not affect the amount of cell surface E-cadherin in EL cells.

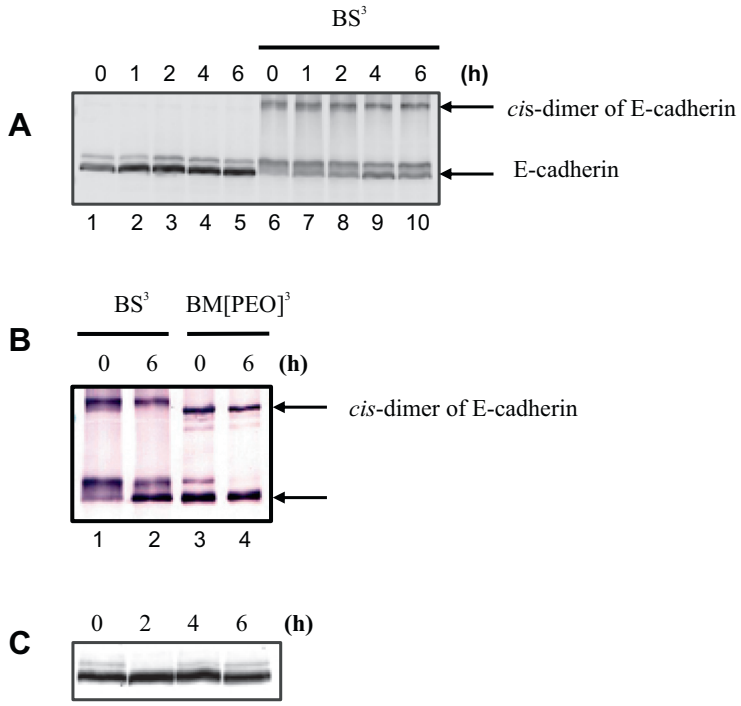

Fig. 2. Effects of $CdCl_2$ on the E-cadherin *cis*-dimer in EL cells. (A) EL cells that were pretreated with medium containing 100μ M $CdCl_2$ for the indicated times were labeled with 5 mM BS^3. The total cell lysates were immunoblotted with the E-cadherin-specific antibody. (B) EL cells that were pretreated with medium containing 100 μM $CdCl_2$ for the indicated times were treated with BS^3 (lanes 1 and 2) and 1 mM $PM[BEO]^3$ (lanes 3 and 4). The total cell lysates were subjected to immunoblotting with anti-E-cadherin. (C) The amount of biotinylated E-cadherin before or after adding 100 μM $CdCl_2$.

3.4. Calcium and cadmium switch assay

We have previously demonstrated that Ca^{2+} induces E-cadherin rigidification and *cis*-dimer formation independent of cell–cell adhesion in EL cells with Ca^{2+}-switch assays that were linked with chemical cross-linking [14]. For the Ca^{2+}-switch assay, EL cells were initially grown to confluence in normal Ca^{2+} growth medium that was subsequently changed to low-Ca^{2+} medium for 12 h, and then the cell–cell adhesion was induced by the addition of Ca^{2+}.

Here, we used a "Cd^{2+}-switch" assay to analyze whether Cd^{2+}-induced E-cadherin *cis*-dimer formation occurred in EL cells that were cultured at low Ca^{2+} concentrations. As expected for a Ca^{2+} antagonist, Cd^{2+} disrupted cadherin function by displacing Ca^{2+} from its binding sites on the extracellular region of cadherin [26]. In our "Cd^{2+}-switch" experiments, we did not find that Cd^{2+}-induced E-cadherin *cis*-dimer formation in the EL cells that were incubated with low-Ca^{2+} medium containing $CdCl_2$ (50 or 100 μM) for 4 h (Fig. 3: lanes 4 and 5). As a control, the Ca^{2+}-induced E-cadherin *cis*-dimer formation was found to act in a Ca^{2+}-concentration-dependent manner (Fig. 3: lanes 1–3). These results were unexpected because Cd^{2+} is a potent Ca^{2+} agonist, and it triggered the formation of stable cadherin dimers on the bead surface [24].

In order to examine the pattern of localization of E-cadherin in EL cells during Cd^{2+} switching, an immunofluoresence analysis was performed. As shown in Fig. 3B, the induction of cell–cell contacts was not found by adding Cd^{2+}, and the cells maintained a distinct rounded morphology during the Cd^{2+} switch. These effects resembled those that occurred when the cells were incubated in the presence of the Ca^{2+} chelator [17]. Immunofluorescence staining revealed that E-cadherin was redistributed from the cell surface into cytoplasmic vesicles (Fig. 3B).

4. Discussion

In the present study, we demonstrated for the first time the effects of Cd^{2+} acting as a Ca^{2+} antagonist on the *cis*-dimer structure of E-cadherin in living cells with a chemical cross-link analysis. In general, the results of the present study were consistent with previous findings [27,15] that suggested that the Cd^{2+}-induced disruption of cell–cell adhesion was associated with the re-localization of E-cadherin from cell–cell borders. In our observations, Cd^{2+}-treated cells exhibited a rapid loss of cell adhesion properties, as shown in Fig. 1A. However, a rapid disappearance of E-cadherin from the cell surface was not observed in EL cells after 6 h of exposure to 100 μM Cd^{2+} (Fig. 2C).

Interestingly, the immunofluorescence data showed that E-cadherin coalesced into punctate aggregates (arrows in Fig. 1B and D) on the cell surface following Cd^{2+} treatment. Recent studies have indicated that the cadherin internalization increases when cell–cell contacts are broken by chelating extracellular Ca^{2+} [10]. Therefore, our results suggested that Cd^{2+} treatment may stabilize cadherin by preventing endocytosis, thereby prolonging its residence time on the EL cell surface. Further studies are required in order to understand the mechanism of the Cd^{2+}-mediated inhibition of cadherin endocytosis after the loss of cell adhesion.

Recent studies have indicated that some pools of cadherin on the cell surface are endocytosed and recycled back to the plasma membrane [28]. This endocytosis has been proposed as a mechanism for downregulating or recycling cadherins, and it has been postulated to play a crucial role in the dynamic regulation of cell adhesiveness. Therefore, our methods with Cd^{2+} acting as a Ca^{2+} antagonist may be useful for understanding the relationships between cadherin endocytosis and adhesion.

Numerous studies have suggested that Cd^{2+} may disrupt cadherin function by displacing Ca^{2+} from its binding sites on the cadherin molecules [15,11]. Moreover, other studies have also shown that Cd^{2+} is able to change the conformation of E-cadherin in epidermal cells [26]. However, we could not find significant changes in the amount of cross-linked and intact E-cadherin at the cell surface with $BM[PEO]^3$ and BS^3 in the presence of 100 μM $CdCl_2$. These results suggested that the conformational changes in the

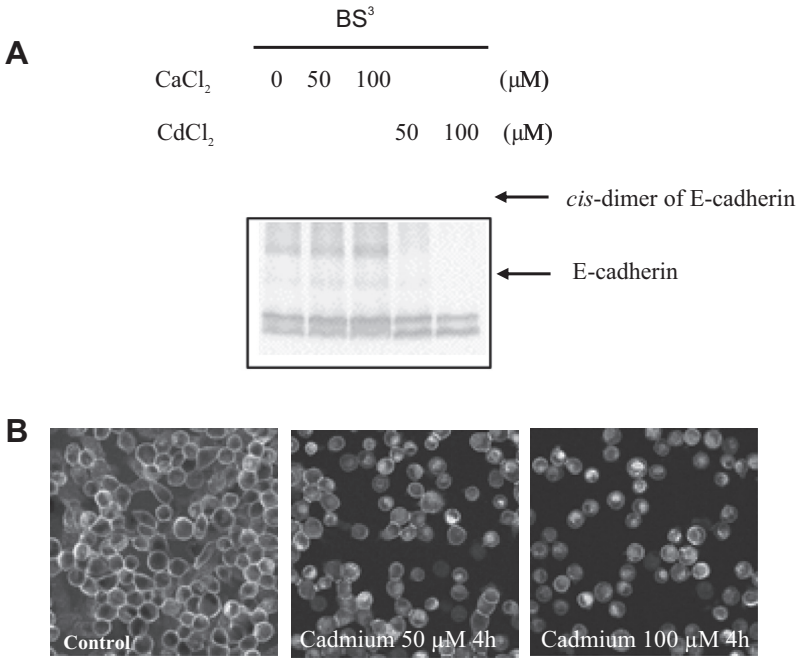

Fig. 3. Calcium and cadmium switch assay. (A) EL cells were cultured in DMEM medium, and the medium was replaced with calcium-free medium. On the following day, the medium was replaced with medium containing 50 or 100 μM $CdCl_2$ for 4 h. The cells were cross-linked with BS^3 and then solubilized (lanes 4 and 5). Control EL cells that were incubated with medium containing 50 or 100 μM $CaCl_2$ for 4 h were also solubilized (lanes 1–3). The total cell lysates were immunoblotted with the E-cadherin-specific antibody. (B) Immunofluorescence microscopy of the E-cadherin pattern during the cadmium switch. EL cells were allowed to attach onto coverglass and the medium was replaced with calcium-free medium. On the following day, the medium was replaced with medium containing 50 or 100 μM $CdCl_2$. The cells were fixed and stained with specific antibodies against E-cadherin.

E-cadherin extracellular domain that were induced by the displacement of Ca^{2+} to Cd^{2+} could affect the adhesive properties, but that this displacement was insufficient to perturb the *cis*-dimer structures of E-cadherin in EL cells that were cultured in normal Ca^{2+} conditions (1.2 mM). However, as shown in the Cd^{2+} switch assay, we did not find that Cd^{2+}-induced E-cadherin *cis*-dimer formation in EL cells that were incubated in low-Ca^{2+} medium containing $CdCl_2$ (50 or 100 μM) for 4 h (Fig. 3).

We have previously shown that Ca^{2+} induces E-cadherin *cis*-dimer formation independent of cell–cell adhesion in EL cells with a Ca^{2+}-switch assay [14]. However, in our Cd^{2+}-switch experiments, we did not find that Cd^{2+}-induced E-cadherin *cis*-dimer formation in EL cells incubated in low-Ca^{2+} medium. One possible explanation for the results in the "Cd^{2+}-switch" assay may be that the Ca^{2+} concentration-dependent competitive displacement of Ca^{2+} by Cd^{2+} from E-cadherin may affect rigidification and its *cis*-dimer structure. In the low Ca^{2+} concentration, Cd^{2+} may be effective in displacing Ca^{2+} from cadherin and, thus promoting a disturbance in its rigidification and *cis*-dimer formation, as shown in Fig. 3. However, in the presence of normal Ca^{2+}, Cd^{2+} may not be effective in displacing Ca^{2+} and producing the structural changes that are produced by Ca^{2+} binding, as shown in Fig. 2.

Troyanovsky et al. have reported that Cd^{2+} triggered the formation of stable cadherin-dimers that were unable to dissociate upon Ca^{2+} depletion on the bead surface *in vitro* [24]. One possible explanation for this apparent discrepancy between the findings of Troyanovsky et al. and our studies may be the use of intact cadherin on the cell surface rather than the myc-tagged form of the cadherin ectodomain on the bead surface system. That is to say, this discrepancy may be due to the Ca^{2+}-mediated structural cooperativity and "allostery" [29] in the native E-cadherin molecule on the cell surface.

Although our data suggested that the Ca^{2+} concentration-dependent competitive displacement of Ca^{2+} by Cd^{2+} from E-cadherin may affect rigidification and its *cis*-dimer structure, additional studies are required to identify the specific structural and conformational effects of Cd^{2+}.

However, our data also showed that Cd^{2+} did not affect the amount of E-cadherin on the cell surface regardless of the disruption in cell–cell adhesion. This observation suggested that Cd^{2+} treatment may lead to the disruption of the cadherin endocytosis pathway in EL cells.

Because the modulation of cadherin availability at the cell surface has emerged as a key factor that determines adhesion strength and because cadherin endocytosis can drive junction disassembly, understanding the cadherin endocytosis pathway is necessary for understanding the dynamic regulation of cell adhesion. Thus, our method is a useful alternative in the study of the relationships between cadherin endocytosis and adhesion *in vivo*.

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