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Calcium influx triggers the sequential proteolysis of extracellular and cytoplasmic domains of E-cadherin, leading to loss of β-catenin from cell–cell contacts

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Cadherins are major cell–cell adhesion molecules in both tumor and normal tissues. Although serum levels of soluble E-cadherin have been shown to be higher in the cancer patients than in healthy volunteers, the detail mechanism regulating release of soluble E-cadherin remains to be elucidated. Here we show that the ectodomain of E-cadherin is proteolytically cleaved from some cancer cells by a membrane-bound metalloprotease to yield soluble form, and the residual membrane-tethered cleavage product is subsequently degraded by intracellular proteolytic pathway. Futhermore, we show that extracellular calcium influx, that is induced by mechanical scraping of cells or ionomycin treatment, enhances the metalloprotease-mediated E-cadherin cleavage and the subsequent degradation of the cytoplasmic domain. Immunocytochemical analysis demonstrates that the sequential proteolysis of E-cadherin triggered by the calcium influx results in translocation of β-catenin from the cell–cell contacts to cytoplasm. Our data suggest that calcium influx-induced proteolysis of E-cadherin not only disrupts the cell–cell adhesion but also activates β-catenin-mediated intracellular signaling pathway, potentially leading to alterations in motility and proliferation activity of cells.

Keywords: metalloprotease; mechanical stimulation; calcium ionophore; E-cadherin; β-catenin

Introduction

Many membrane-anchored proteins, including growth factor receptors, ligands, cell-adhesion molecules, and enzymes are released from cells by various mechanisms (Gordon, 1991). One key mechanism to generate the soluble form is proteolytic processing of the extracellular domain (ectodomain) of such membrane proteins (Hooper *et al*., 1997). Proteolytic degradation of cell adhesion molecules and release of bioactive ligands and receptors can mediate rapid responses to changes in the cellular microenvironment and exert significant influence on morphogenesis, cell fate specification, cell migration, tissue repair, and apoptosis.

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It has been reported that proteolysis of the ectodomain of some membrane-anchored proteins is activated in cancer cells. In colon cancer cells, the soluble TNFα receptor has been found yielded into culture supernatants through metalloprotease-mediated proteolytic cleavage. The down-regulation of the TNFα receptor at the cell surface by cleavage has been proposed to suppress the apoptosis of tumor cells mediated through the 'death domain' of TNFα-RI (Lombard *et al*., 1998). In breast cancer cells, the HER2/neu proto-oncogene product has been shown to undergo proteolytic cleavage of its ectodomain by metalloprotease and the amino-terminal truncation of HER2/neu protein by the cleavage is suggested to enhance tyrosine kinase activity and transforming efficiency (Zabrecky *et al*., 1991; Lin and Clinton, 1991; Codony-Servat *et al*., 1999; Di Fiore *et al*., 1987). Moreover, we have recently demonstrated that the ectodomain of adhesion molecule CD44 is proteolytically cleaved by membrane-associated metalloprotease in various cancer cells and that this cleavage plays a critical role in CD44-mediated tumor cell migration (Okamoto *et al*., 1999). These findings suggest that the activation of processing of membrane-anchored proteins in cancer cells alters the cellular microenvironment, which is advantageous to their growth and invasion.

E-cadherin plays an essential role in the maintenance of cell–cell adhesion of epithelial cells by homophilic interaction (Takeichi, 1991). The E-cadherin-mediated cell adhesion is often disorganized in tumor cells, and that is potentially a cause of the unregulated behavior of tumor cells, including invasion and metastasis (Takeichi, 1993; Hirohashi, 1998). Soluble forms of E-cadherin were firstly detected as 80–84 kD peptides released from the MCF-7 human breast cancer cells (Damsky *et al*., 1983). More recently, serum levels of soluble E-cadherin have been shown to be significantly elevated in cancer patients (Katayama *et al*., 1994; Gofuku *et al*., 1998). However, the mechanism regulating release of soluble E-cadherin in cancer cells remains to be elucidated. Ectopic expression of stromelysin-1, which is a soluble matrix metalloprotease (MMP), in non-cancerous mammary epithelial cells resulted in the release of soluble E-cadherin (Lochter *et al*., 1997), implying that metalloproteases are involved in the shedding of E-cadherin. However, most of the cancer cell lines examined, including MCF-7 cells, expressed no measurable amounts of stromelysin-1 (Sato *et al*., 1992; Ito *et al*., 1995). These findings

raise the question as to how the release of soluble E-cadherin is actually regulated in cancer cells.

To address this question, we have examined the mechanism and biological significance of the secretion of soluble E-cadherin. We demonstrate that E-cadherin is constitutively cleaved at membrane-proximal region by a membrane-associated metalloprotease in some cancer cell lines and that the metalloprotease-mediated E-cadherin cleavage is dramatically enhanced by mechanical stimulation which induces extracellular calcium influx. We further present that the membrane-tethered cleavage products left after cleavage are rapidly degraded through an intracellular proteolytic pathway, and that this sequential proteolysis of E-cadherin leads to the translocation of β-catenin, which is a binding partner of E-cadherin, from the plasma membrane to cytoplasm. These results suggest that activation of β-catenin-mediated intracellular signaling pathway triggered by E-cadherin proteolysis contributes to movement and proliferation of cells after mechanical stimulation.

Results

Involvement of metalloproteases in the release of soluble E-cadherin from various cancer cells

First, we examined the levels of soluble E-cadherin in the culture supernatants of various cancer cell lines. Four thousand cells were cultured in serum-free medium for 24 h, and the supernatants were analysed for soluble E-cadherin by an ELISA system. As shown in Figure 1a, the levels of soluble E-cadherin released into the culture supernatant varied depending on the cell line (Figure 1a).

To determine whether the soluble E-cadherin are generated from proteolytic cleavage at the surface of cancer cells, we examined the effects of various protease inhibitors on the release of soluble E-cadherin by the ELISA analysis. Treatment of A431 cells with hydroxamic acid based metalloprotease inhibitors, BB2516 and CGS27023A (Talbot and Brown, 1996), significantly reduced the amounts of soluble E-cadherin (Figure 1b). However, the serine protease inhibitors (3,4-DCI, TLCK, and aprotinin), the aspartic acid protease inhibitor (pepstatin A) and the cysteine protease inhibitor (E-64) did not inhibit the release of soluble E-cadherin. These results suggest that the soluble E-cadherin is yielded by proteolytic cleavage of E-cadherin at the cell surface and that a member of the metalloprotease family is involved in the cleavage in cancer cells.

Rapid degradation of membrane-associated E-cadherin cleavage products

Since E-cadherin is likely to be cleaved by a metalloprotease at the cell surface as shown above, this cleavage event could produce membrane-associated cleavage products which consist of the membrane-spanning domain and the cytoplasmic domain of E-cadherin. To detect the membrane-associated cleavage products of E-cadherin, we performed Western blot analysis using a monoclonal antibody that recognizes the cytoplasmic domain of E-cadherin (referred as anti-E-CDcyto Ab). Although A431,TE12 and TE13 cells were shown to secrete relatively high amounts of soluble E-cadherin, only the band of full-length E-cadherin was detected, but the band corresponding to the membrane-associated cleavage product was not clearly identified (Figure 2a, lanes 1, 3 and 5). However, in the presence of MG132, which inhibits intracellular proteolysis activities including the proteasome, calpains and lysosomal cysteine proteases, anti E-CDcyto Ab detected a 33 kD band, which is a possible membrane-associated cleavage product, in addition to the full length E-cadherin (Figure 2a, lanes 2, 4 and 6). Additionally, the 33 kD bands were hardly detectable in cell lines which release low amounts of soluble E-cadherin (A549 and SW480)

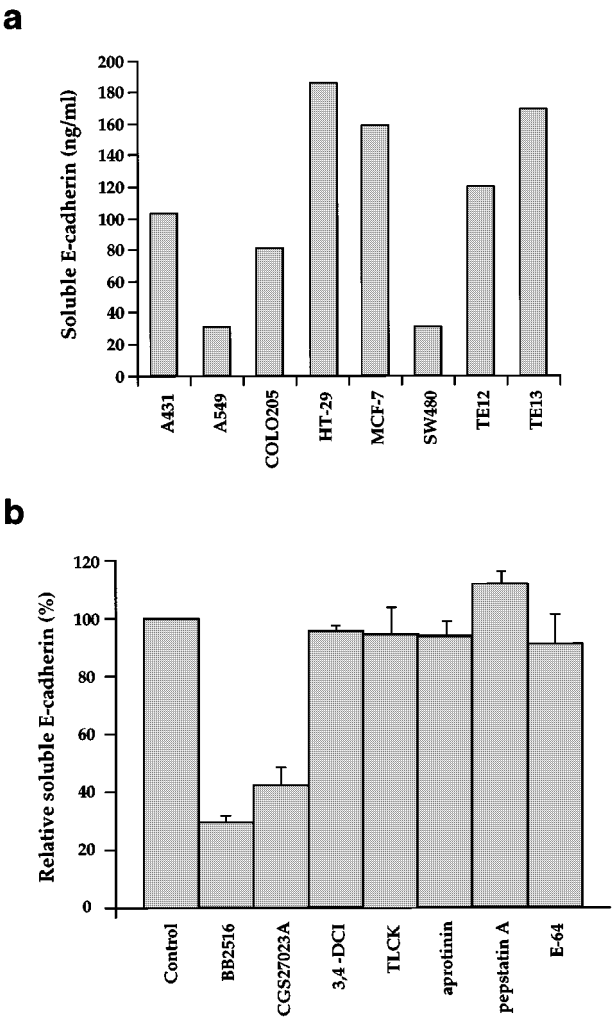


Figure 1 Detection of soluble E-cadherin by the ELISA system. (**a**) Soluble E-cadherin in culture supernatants of various cancer cell lines. Four thousand cells were cultured in serum-free medium for 24 h, and soluble E-cadherin in the cell-free supernatants was detected by ELISA. (**b**) Effect of protease inhibitors on the release of soluble E-cadherin. A431 cells were incubated for 12 h at 37°C in the absence (control) or presence of various protease inhibitors, as indicated. Final concentrations of the agents added were as follows: BB2516 (marimastat), 100 μM; CGS27023A, 100 μM; 3,4-dichloroisocoumarin (DCI), 100 μM; N α-p-tosyl-L-lysine chloromethyl ketone (TLCK), 0.2 μg/ml; aprotinin, 2 μg/ml; pepstatin A, 100 μM; trans-Epoxysuccinyl-L-leucylamido(4-guanidino) butane (E-64), 10 μM and the cell-free supernatants were analysed for soluble E-cadherin using an ELISA system. Columns and bars represent mean and s.d. obtained from three independent experiments

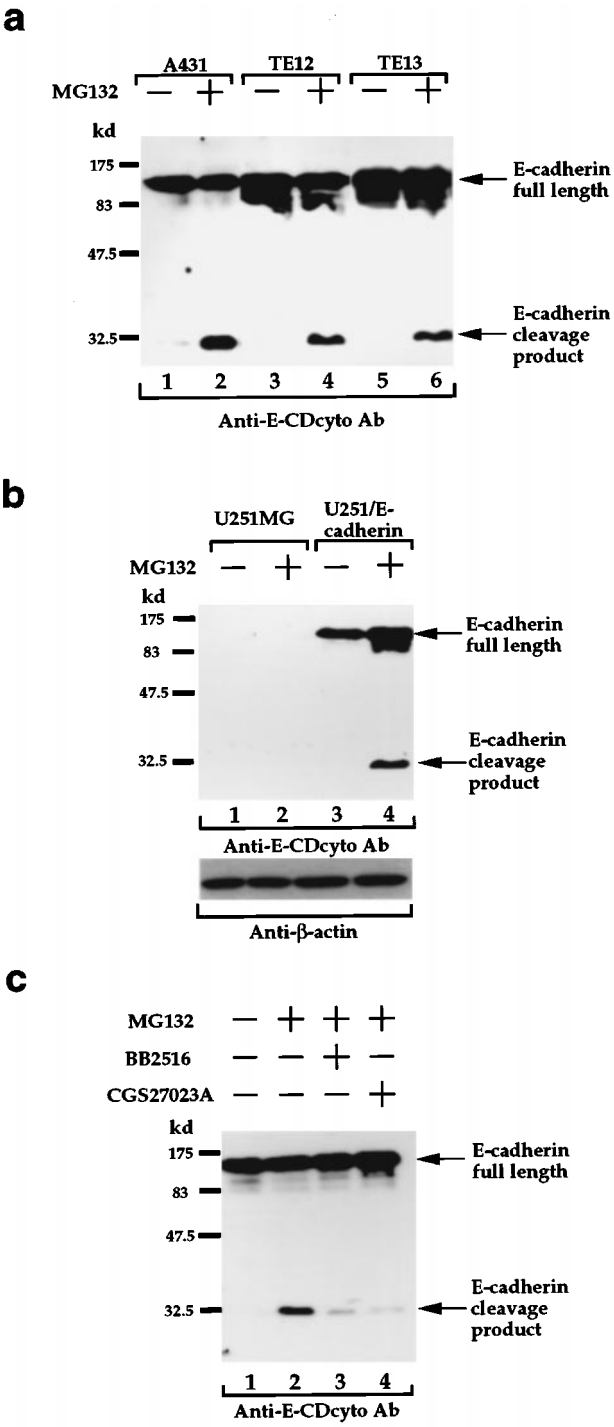


Figure 2 Detection of the 33 kD E-cadherin cleavage product by anti-E-CDcyto Ab. (**a**) Western blot analysis of E-cadherin cleavage in A431, TE12, or TE13 cells. Cell lines were incubated for 8 h at 37°C in the absence (−) or presence (+) of MG132 (10 μM) and analysed by Western blotting using anti-E-CDcyto Ab. (**b**) Western blot analysis of E-cadherin expression in U251MG cells and E-cadherin transfected U251MG (U251/E-cadherin) cells. U251MG and U251/E-cadherin cells were incubated for 8 h at 37°C in the absence (−) or presence (+) of 10 μM MG132. Cell lysates were analysed by Western blot using anti-E-CDcyto Ab (upper panel) or anti-β-actin Ab (lower panel). (**c**) Effect of metalloprotease inhibitors on E-cadherin cleavage. A431 cells were treated with no agent (lane 1), 10 μM MG132 (lane 2), 10 μM MG132 and 100 μM BB2516 (lane 3) or 10 μM MG132 and 100 μM CGS27023A (lane 4) for 8 h at 37°C. Cell lysates were analysed by Western blot using anti-E-CDcyto Ab

even when the cells were treated by MG132 (data not shown).

To determine whether the 33 kD bands are derivatives of E-cadherin, we transfected U251MG glioma cells, which were determined not to have the detectable endogenous E-cadherin (Figure 2b, lane 1), with the E-cadherin expression plasmid and established a stable line expressing E-cadherin. The cell line, termed U251/E-cadherin, was analysed for ectopically expressed E-cadherin protein by Western blot using anti-E-CDcyto Ab. In the lysates of MG132-treated U251/E-cadherin, the 33 kD band, in addition to the full-length E-cadherin, was detected (Figure 2b, lane 4), whereas no bands were detected in the parental U251MG cells (Figure 2b, lane 2). Furthermore, U251/E-cadherin cells without MG132 treatment expressed only the full-length E-cadherin and not the 33 kD species (Figure 2b, lane 3). Since the 33 kD species, as well as the full length E-cadherin, were detected in the membrane fraction washed with 1 M NaCl (data not shown), the species are considered to anchor to the membrane by a transmembrane domain. The molecular weight of the species was greater than the predicted size of E-cadherin transmembrane and cytoplasmic domains, indicating that the 33 kD species are the membrane-associated cleavage product left after cleavage of the ectodomain of E-cadherin.

When MG132-treated A431 cells were cultured in the presence of metalloprotease inhibitor, BB2516 or CGS27023A, the 33 kD band became hardly detectable (Figure 2c). In contrast, the 33 kD band was clearly detected in the MG132-treated A431 cells in the presence of the serine protease inhibitor (3,4-DCI), the aspartic acid protease inhibitor (pepstatin A) and the cysteine protease inhibitor (E-64) (data not shown). These findings, consistent with the ELISA data, indicate that the 33 kD band detected with anti-E-CDcyto Ab is a proteolytic product yielded as a result of metalloprotease-mediated E-cadherin cleavage. Taken together, our observations suggest that E-cadherin is cleaved by the metalloproteases at the ectodomain, and the remnant 33 kD product is rapidly degraded by an intracellular proteolytic pathway.

Identification of the E-cadherin cleavage site at the ectodomain

To determine the site which is cleaved by the metalloprotease, the 33 kD E-cadherin cleavage product was purified from A431 cell lysates with affinity chromatography using anti-E-CDcyto Ab immobilized agarose column and subjected to automated N-terminal amino acid sequence analysis. The Edman degradation of the purified 33 kD protein yielded two sequences, one was SDVND and the other was DVNDN. In comparing these data with the sequence of E-cadherin ectodomain, two sequential sites, Ser(582)-Asp(586) and Asp(583)-Asn(537), were found to be identical to the N-terminal sequences of the 33 kD E-cadherin product. The latter sequence may be generated by the cleavage taken place at one residue downstream of the former one. These data suggest the likely cleavage point to be at Lue(581)-Ser(582) and/or Ser(582)-Asp(583), which is 121 and/or 120 amino acid distal from the transmembrane domain (Figure 3). Therefore, the membrane-tethered cleavage product is

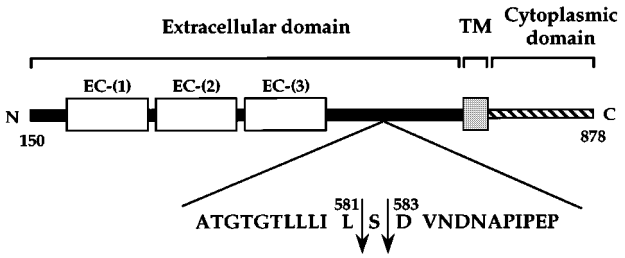


Figure 3 Schematic representation of the metalloprotease-mediated cleavage region of E-cadherin. The amino acid sequence of E-cadherin near the cleavage sites is shown. Arrows locate the proposed cleavage sites determined by amino-terminal amino acid sequence analysis of the cleavage products. EC-(1), (2) and (3) indicate E-cadherin internal sequence repeats at the extracellular domain. TM indicates transmembrane domain

expected to contain 297 (or 296) amino acid residues, with a calculated molecular mass of 32938 (or 32851), which is close to its apparent M_r of 33 kD estimated by SDS–PAGE.

Cleavage of E-cadherin at the ectodomain in cancer cells by a membrane-associated metalloprotease

We examined whether the metalloprotease responsible for cleavage of E-cadherin ectodomain is a soluble secreted enzyme or a membrane-bound enzyme. In the presence of MG132, we first incubated SW480 cells (5×10^5 cells), which have low E-cadherin cleavage activity, in the culture supernatants of HT-29 cells (5×10^5 cells) which have high E-cadherin activity. The 33 kD E-cadherin cleavage products was not clearly identified in cell lysates extracted from the treated SW480 cells by Western blot analysis using anti E-CDcyto Ab (Figure 4a). These results suggest that the metalloprotease involved in E-cadherin cleavage is not a soluble, secreted enzyme.

To investigate whether the protease has a membrane-associated activity, we utilized cell-free E-cadherin cleavage assays. Crude membranes were isolated from U251/E-cadherin cells followed by washed with buffer containing 1 M NaCl. The extracted membrane fraction were incubated at 37°C for 120 min and then analysed by Western blot using anti-E-CDcyto Ab. The 33 kD cleavage product markedly increased compared to the preincubated control sample, and concomitantly the full-length 120 kD E-cadherin completely disappeared (Figure 4b, lanes 1 and 2). Furthermore, the *in vitro* E-cadherin cleavage reaction was blocked by BB2516 (Figure 4b, lane 3). These results indicate that E-cadherin can be cleaved *in vitro* under the conditions we utilized and that the responsible protease is most likely to be a membrane-associated metalloprotease.

Extracellular calcium influx induces E-cadherin ectodomain cleavage followed by intracellular degradation of the cleavage product

In the course of our Western blot analysis, we found that the 33 kD E-cadherin cleavage product was clearly detected with anti-E-CDcyto Ab when the lysates were prepared from the cells mechanically scraped without MG132 treatment (Figure 5a, lane 2). The E-cadherin

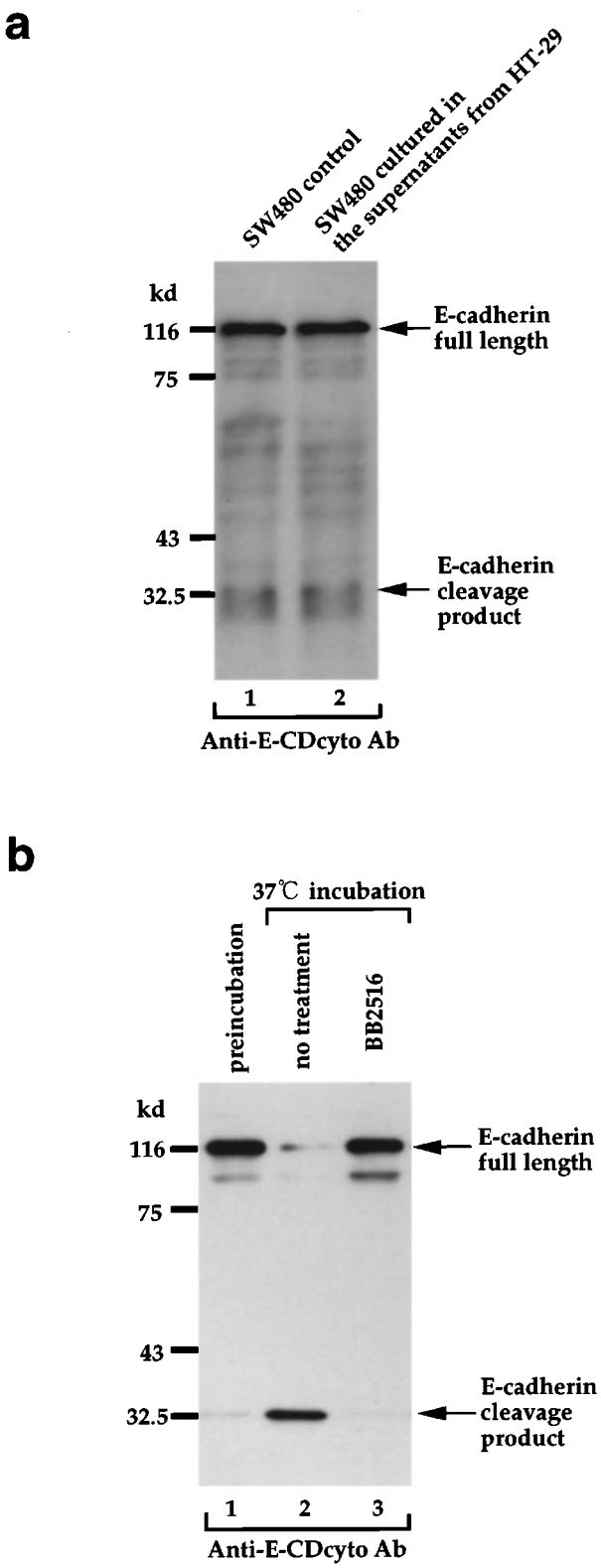


Figure 4 Localization of the metalloprotease activity involved in E-cadherin cleavage. (**a**) Analysis of E-cadherin cleavage activity in the culture supernatants. In the presence of MG132 (10 μM), SW480 cells (5×10^5 cells) were incubated for 12 h with flesh medium (lane 1) or the cell-free supernatants from HT-29 cells (lane 2). Cell lysates were analysed by Western blot using anti-E-CDcyto Ab. (**b**) Analysis of the E-cadherin cleavage activity in membrane fraction. U251/E-cadherin membrane fractions were washed with the ice-cold Tris-HCl (30 mM, pH 7.2) containing 1 M NaCl. The washed membrane fractions reconstituted in the ice-cold Tris-HCl (30 mM, pH 7.2) were either immediately mixed with SDS sample buffer (lane 1) or incubated at 37°C for 2 h with no agents (lane 2), 100 μM BB2516 (lane 3). Each aliquot was subjected to Western blot using anti-E-CDcyto Ab

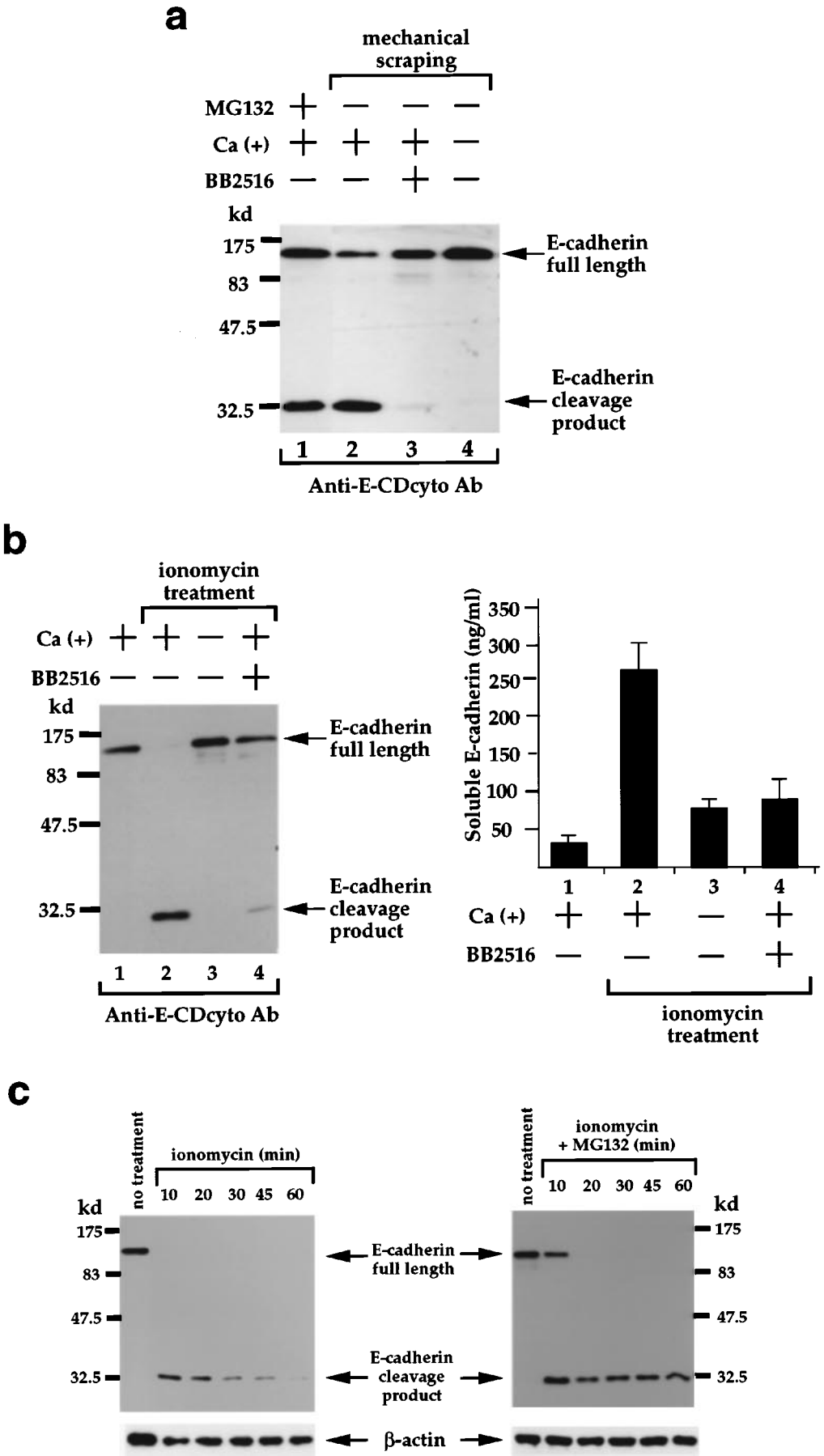


Figure 5 Enhancement of the E-cadherin cleavage by mechanical stimulation and extracellular calcium influx. (**a**) Effect of mechanical scraping of A431 cells on the metalloprotease-mediated E-cadherin cleavage. MG132-treated A431cells were directly lysed with SDS sample buffer (lane 1). A431 cells in TBS with 2 mM $CaCl_2$ (lane 2), TBS with 2 mM $CaCl_2$ containing 100 μM BB2516 (lane 3) and TBS without $CaCl_2$ and BB2516 (lane 4) were mechanically scraped and the cell pellets were lysed with SDS

cleavage product was hardly detected in the presence of BB2516 even when cells were mechanically scraped (Figure 5a, lane 3), consistent with the notion that E-cadherin proteolytic cleavage is evoked by metalloproteases. These results suggest that the mechanical scraping of cells enhance the metalloprotease-mediated proteolytic cleavage of E-cadherin.

It has been reported that mechanical stimulations, such as scraping of cells, elevate the intracellular Ca^{2+} concentration and trigger various cellular responses. Therefore, we speculated that the increase in intracellular Ca^{2+} that resulted from mechanical scraping of cells could enhance proteolytic cleavage of E-cadherin. To examine this possibility, the cultured cells were mechanically scraped in the presence or absence of extracellular Ca^{2+} and subjected to Western blot analysis using anti-E-CDcyto Ab. In the cell lysates prepared in the absence of extracellular Ca^{2+}, the 33 kD E-cadherin cleavage bands were not detected (Figure 5a, lane 4), suggesting that the enhancement of E-cadherin cleavage during the mechanically scrape is necessary for extracellular Ca^{2+} influx. Based on this finding, we treated TE13 cells with the calcium ionophore ionomycin without the scraping and examined the effects on E-cadherin cleavage by both Western blotting, for E-cadherin cleavage product, and ELISA for soluble E-cadherin. Treatment with ionomycin increased both the E-cadherin cleavage product and soluble E-cadherin (Figure 5b). In addition, ionomycin induced E-cadherin cleavage was abolished either in Ca^{2+}-free condition or in the presence of BB2516. These results indicate that the influx of Ca^{2+} across the plasma membrane enhances the metalloprotease-mediated E-cadherin cleavage.

As shown in Figure 5c, the amount of the 33 kD cleavage product yielded by ionomycin treatment was reduced in a time-dependent manner and the reduction of the cleavage product was completely blocked by MG132. These results strongly supports the notion that the cleavage product resulting from the E-cadherin ectodomain cleavage is degraded through intracellular proteolytic pathways.

Translocation of β-catenin from the membrane to cytoplasm by the sequential proteolytic processing of E-cadherin

β-catenin is known to interact with the cytoplasmic domain of E-cadherin and links it to the actin cytoskeleton through α-catenin. As demonstrated above, E-cadherin is cleaved at the ectodomain by metalloprotease and the membrane-tethered cleavage product is subsequently degraded. Therefore, we next examined whether the subcellular localization of β-catenin is altered when the sequential proteolysis of E-cadherin is promoted by ionomycin treatment. Immunocytochemical analysis of TE13 cells using monoclonal antibodies against the ectodomain (termed HECD-1), the cytoplasmic domain of E-cadherin (anti-E-CDcyto Ab) and β-catenin showed that both E-cadherin and β-catenin were present at cell–cell junctions when cells were not treated with any agents (Figure 6a (i)). However, by ionomycin treatment for 45 min there was a marked decrease in both E-cadherin and β-catenin at cell–cell junctions and β-catenin was diffusely distributed inside the cells (Figure 6a (ii)). This ionomycin-induced loss of E-cadherin and β-catenin from plasma membrane was significantly suppressed by treatment with metalloprotease inhibitor BB2516 (Figure 6a (iii)). Furthermore, when cells were treated with MG132 to block the intracellular proteolysis, both cytoplasmic domain of E-cadherin and β-catenin were still present at cell–cell junctions, whereas the E-cadherin ectodomain was not detected, presumably due to the cleavage at the cell surface (Figure 6a (iv)).

For an additional approach to confirm the translocation of β-catenin from plasma membrane to cytoplasm, we investigated a time-dependent movement of β-catenin after the ionomycin treatment by both immunocytochemistry and Western blot analyses. As shown in Figure 6b, β-catenin localized to plasma membrane became diffusely distributed inside the cell by ionomycin treatment in a highly dynamic manner. Consistent with this finding, Western blot analysis clearly demonstrated that ionomycin treatment induced translocation of β-catenin from membrane to cytoplasmic fraction (Figure 6c). These findings suggest that the metalloprotease-mediated cleavage of the E-cadherin ectodomain takes place prior to proteolysis of the membrane-tethered cleavage product and that this sequential proteolysis of E-cadherin, which is enhanced by calcium influx, gives rise to the translocation of β-catenin from cell–cell junctions to cytoplasm.

Discussion

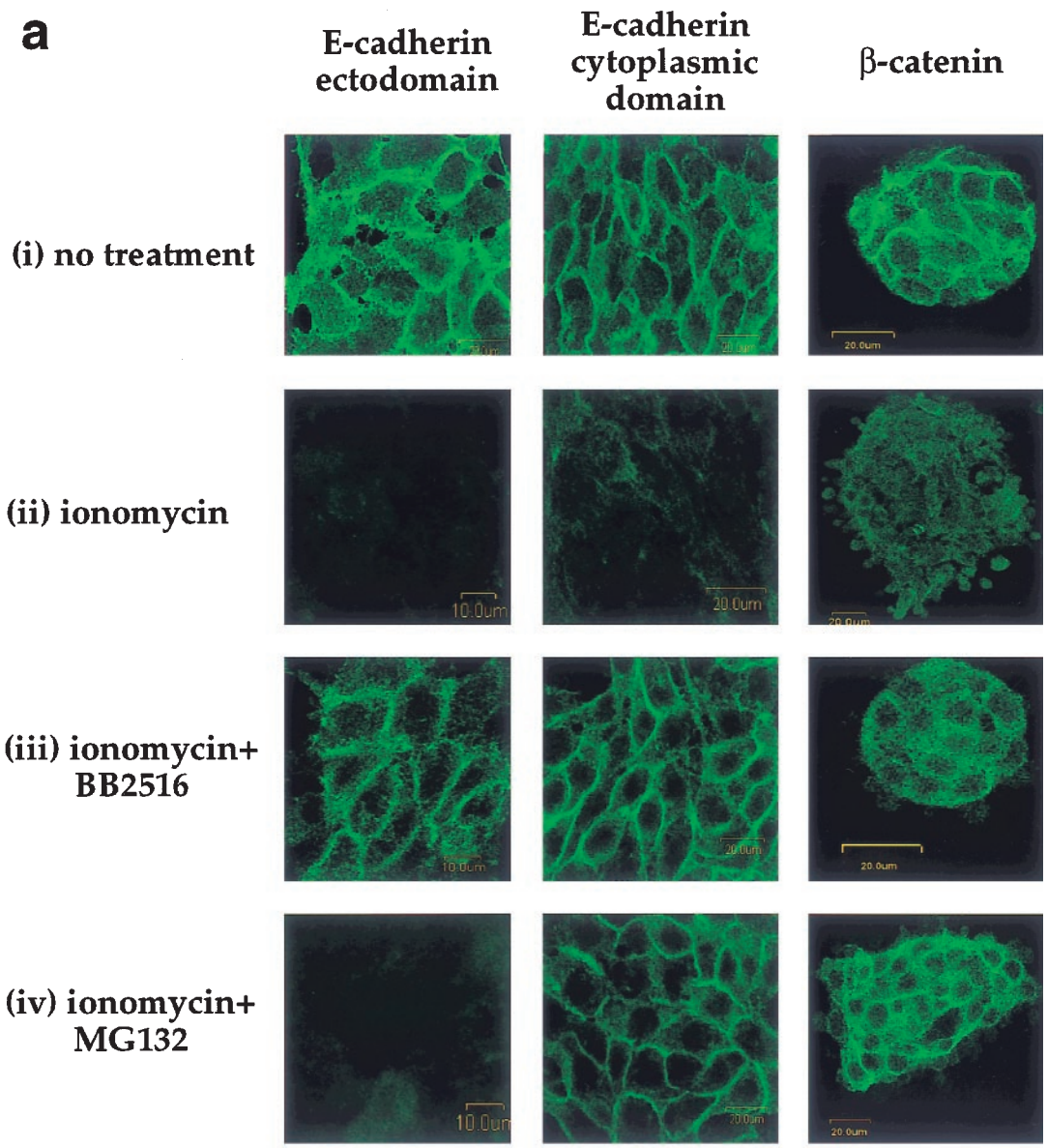
Our interest in earlier findings that the amount of soluble E-cadherin in serum is higher in the cancer patients than in healthy volunteers (Katayama *et al*., 1994; Gofuku *et al*., 1998) stimulated the analysis presented here on the mechanism regulating release of soluble E-cadherin and its resultant biological responses. We first examined soluble E-cadherin in the cultured supernatant of various cancer cell lines by an ELISA system and demonstrated that several cancer cell lines secrete relatively high amounts of soluble E-cadherin. E-cadherin expressed in mammary epithelial cells has been shown to be proteolytically cleaved by ectopically-expressed stromelysin-1, which is a MMP family protein (Lochter *et al*., 1997). Moreover, the soluble form of vascular endothelial-

sample buffer. Cell lysates were analysed by Western blot using anti-E-CDcyto Ab. (**b**) Effect of calcium influx on the E-cadherin cleavage. TE13 cells were treated with no agent (lane 1) or 10 μM ionomycin for 20 min at 37°C in TBS with 2 mM $CaCl_2$ (lane 2), TBS without 2 mM $CaCl_2$ (lane 3) and TBS with 2 mM $CaCl_2$ containing 100 μM BB2516 (lane 4). Cell lysates were analysed by Western blot using anti-E-CDcyto Ab (left panel), and the cell-free supernatants were analysed for soluble E-cadherin using an ELISA system (right panel). Columns and bars (right panel) represent mean and s.d. obtained from three independent experiments. (**c**) Degradation of the E-cadherin cleavage product. TE13 cells were treated with ionomycin (10 μM) in either the serum-free medium or the medium containing MG132 at 37°C for various time periods (up to 60 min), and cell lysates were analysed by Western blot using anti-E-CDcyto Ab (upper panels) or anti-β-actin (lower panels)

cadherin (VE-cadherin) has been found to be generated by metalloprotease-mediated proteolytic cleavage during apoptosis in human umbilical vein endothelial cells (HUVEC) (Herren *et al*., 1998). Consistent with these reports, we have demonstrated that treatment with metalloprotease inhibitors reduced the amount of soluble E-cadherin released from cancer cells.

While the soluble E-cadherin has been detected in culture supernatants, the residual membrane-tethered domain left after cleavage has not been detectable by Western blot analysis using anti-E-CDcyto Ab, suggesting degradation of the membrane-tethered cleavage product. As we have speculated, the cleavage product become apparent by treatment with MG132, which inhibits intracellular proteolysis activities including the proteasome, calpains and lysosomal cysteine proteases. We also confirmed that the site of cleavage lies at the membrane-proximal stalk region by N-terminal sequencing of the cleavage product. All these data give evidence that E-cadherin expressed in cancer cells is proteolytically cleaved at the ectodomain via metalloproteases followed by intracellular degradation of the cleavage products.

Although a number of membrane proteins are known to be cleaved at their ectodomains by metalloproteases, the type of metalloprotease has not been classified in most cases. In the present study, our cell-free assay has revealed that a membrane-bound metalloprotease is responsible for E-cadherin cleavage in cancer cells. This result seems to be contradictory to the previous report that ectopically-expressed stromelysin-1, which is a family of soluble MMP, induces the cleavage of E-cadherin in mammary epithelial cells (Lochter *et al*., 1997). However, MCF-7 human mammary carcinoma cells, which secrete large amounts of soluble E-cadherin, have been demonstrated to express no measurable amounts of stromelysin-1 (Ito *et al*., 1995). Therefore, ectopically-expressed stromelysin-1 may be able to activate metalloprotease-mediacted E-cadherin cleavage di-



rectly or indirectly, but not be a participant in physiological E-cadherin cleavage. In fact, expression of stromelysin-1 in mammary epithelial cells has been reported to lead to activation and upregulation of other metalloprotease (Lochter *et al*., 1997). It is possible that stromelysin-1 promotes E-cadherin cleavage by activating other proteases, including membrane-bound metalloproteases. The property of the metalloproteases responsible for E-cadherin cleavage reported here will aid the future purification and identification of the putative protease.

In this study, we have found that extracellular calcium influx, that is induced by mechanical scraping of cells or ionomycin treatment, enhances the metalloprotease-mediated E-cadherin cleavage. Increased intracellular Ca^{2+} level is known to transduce the diverse intracellular signaling (Berridge *et al*., 1998). The proteolytic cleavage of other cell surface molecules, including TGF-α, c-kit receptor and HB-EGF, can also be enhanced by extracellular calcium influx (Pandiella *et al*., 1992; Yee *et al*., 1993; Dethlefsen *et al*., 1998). These data suggest the existence of a common pathway for the induction of proteolytic cleavage through calcium mobility, though the underlying mechanism remains to be elucidated.

The Met tyrosine kinase receptor was reported to be cleaved at the ectodomain and the resulting membrane-associated cleavage product, which could potentially possess oncogenic capability, is degraded by the proteasome (Jeffers *et al*., 1997). Degradation of the Met receptor was proposed to play a significant biological role in desensitization of oncogenic signaling. Our results indicate that the residual membrane-tethered E-cadherin left after the ectodomain cleavage is rapidly degraded by an intracellular proteolytic pathway and that ionomycin-treatment, which enhances the sequential degradation of the E-cadherin, leads to release of β-catenin from the membrane to the cytoplasm. β-

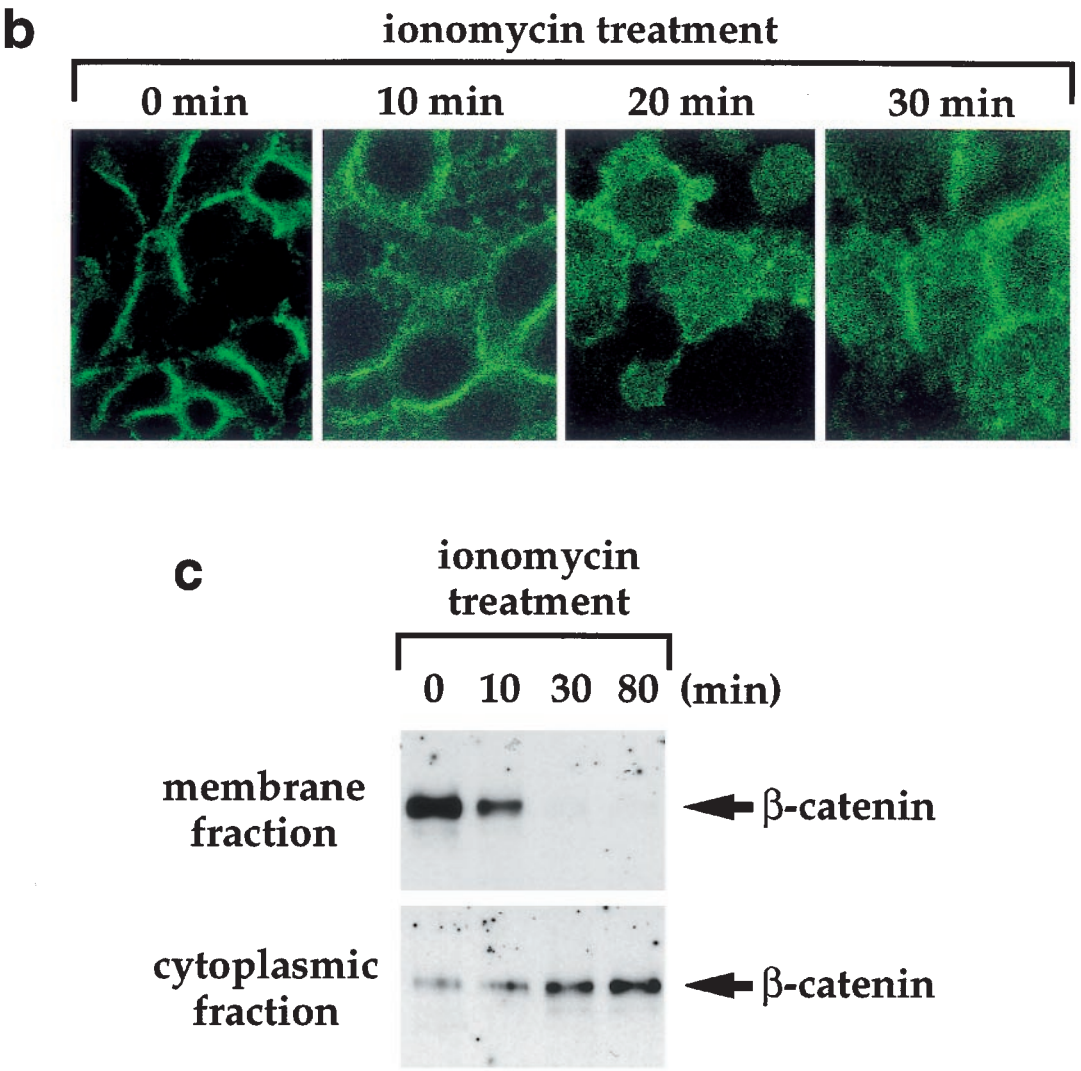


Figure 6 The effect of E-cadherin proteolytic processing on subcellular localization of β-catenin. (**a**) TE13 cells were treated with no agent (i) or 10 μM ionomycin for 45 min in serum-free medium (ii), BB2516 (100 μM) (iii), or the medium containing MG132 (10 μM) (iv). After the treatment, the fixed cells were stained with HECD-1 Ab which recognizes E-cadherin ectodomain (left panel), anti-E-CDcyto Ab (mid panel) or anti-β-catenin Ab (right panel) followed by FITC-conjugated secondary antibody, and analysed by confocal microscopy. (**b**) TE13 cells were treated with 10 μM ionomycin for 0, 10, 20 or 30 min. After the treatment, the fixed cells were stained with anti-β-catenin Ab followed by FITC-conjugated secondary antibody. (**c**) TE13 cells were treated with 10 μM ionomycin for 0, 10, 30, or 80 min, and the membrane and cytoplasmic fractions were immunoblotted with anti-β-catenin Ab

catenin is known to link the cytoplasmic domain of E-cadherin to the actin cytoskeleton via α-catenin and, therefore, is required for establishing E-cadherin-mediated cell–cell adhesion (Ozawa *et al*., 1989; Aberle *et al*., 1994; Hulsken *et al*., 1994). Although β-catenin was initially considered to merely act as an apparatus for cell–cell adhesion, it also has been found to behave as an intracellular signal transduction molecule (Polakis, 1999). The free cytoplasmic β-catenin is involved in two subcellular systems that affect its activities and fate: one is a unique degradation pathway that regulates the level of β-catenin by the ubiquitin-proteasome system; and the other is the transcriptional machinery, where β-catenin interacts with LEF/TCF (lymphoid enhancer factor-1/T-cell factor) transcriptional factors and activates the expression of specific target genes, such as E-cadherin, c-*myc* and cyclin D1 (Huber *et al*., 1996; He *et al*., 1998; Sadot *et al*., 1998; Tetsu and McCormick, 1999). Therefore, we propose that the sequential proteolysis of E-cadherin may be important for triggering a β-catenin-mediated intracellular signaling pathway. Particularly, the calcium influx, mainly caused by mechanical stimulation of the cell membrane, has been identified to drastically induce the extra- and intracellular E-cadherin proteolysis and resulted in rapid loss of β-catenin from the plasma membrane. Therefore, it is reasonable to speculate that calcium influx contributes not only to separation of cell–cell adhesion by E-cadherin ectodomain cleavage, but also to induction of β-catenin/TCF-mediated cellular responses, including alterations in morphology, motility and proliferation activity of cells, by degradation of cytoplasmic domain of E-cadherin followed by translocation of β-catenin to cytoplasm. Experiments to investigate whether the ionomycin treatment or mechanical stimulation induces the β-catenin/TCF-mediated transcriptional activation are in progress.

Mechanically scratching monolayers of cells has been observed to relieve contact inhibition and induce surviving cells near the wound edge to move and proliferate (Coomber and Gotlieb, 1990; Todaro *et al*., 1965). Upon mechanical wounding of quiescent cells, a transient increase in the concentration of intracellular free Ca^{2+} is induced in cells at the wound edge (Tran *et al*., 1999). Furthermore, it was reported that proliferation of vascular smooth muscle cells following balloon catheter injury was significantly reduced by administration of calcium antagonists (Reidy and Jackson, 1990). Therefore, calcium is considered to be one signal that plays a major regulatory role in the stimulation of wound healing. However, it has not been well understood how calcium influx caused by injury stimulates proliferation and motility of cells. The results in our study suggest one potential signaling pathway which contributes to the spatial control of movement and proliferation of cells after tissue injury. Further investigations of the regulatory mechanism of the sequential proteolytic pathway and the functional significance of free cytoplasmic β-catenin after E-cadherin degradation will provide clues to the induction of cell growth after the tissue injury or to the inhibition of tumor cell growth and migration after therapeutic manipulations.

Materials and methods

Cell culture

The human esophageal carcinoma cell lines TE12 and TE13, the colon carcinoma cell lines HT-29, SW480 and Colo205, the mammary carcinoma cell line MCF-7, the lung cancer cell line A549, and the malignant glioma cell line U251MG were grown in Dulbecco's modified Eagle's medium with Ham's F-12 nutrient mixture (DMEM/F12) (Life Technologies, Inc [GIBCO–BRL], Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Bio-Whittaker, Walkersville, MD, USA) at 37°C in an atmosphere containing 5% CO_2. The human epidermoid carcinoma cell line A431 was grown in Dulbecco's modified Eagle's medium with high glucose (Life Technologies, Inc. [GIBCO–BRL]) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in an atmosphere containing 5% CO_2.

Antibodies and reagents

The monoclonal antibodies against β-catenin (referred as anti-β-catenin Ab) and cytoplasmic domain of E-cadherin (referred as anti-E-CDcyto Ab) were purchased from Transduction Laboratories (Lexington, KY, USA). Anti-E-cadherin ectodomain monoclonal antibody (HECD-1) and anti-β-actin monoclonal antibody (AC-15) were purchased from Takara Shuzo (Kyoto, Japan) and Sigma Chemical Co (St. Louis, MO, USA), respectively.

Reagents were obtained as follows: 3,4-dichloroisocoumarin (DCI), N α-p-tosyl-L-lysine chloromethyl ketone (TLCK), trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), pepstatin A, aprotinin from Sigma Chemical Co; calcium ionophore (ionomycin) from Calbiochem (La Jolla, CA, USA); carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132) from Peptide Institute (Osaka, Japan). Hydroxamate-based metalloprotease inhibitor, BB2516 (marimastat) and CGS27023A, were kindly provided by Novartis Pharmaceutical Co (Takarazuka, Japan).

Plasmids and transfection

Total RNA of A431 was isolated by the acid-guanidium thiocynate-phenol-chloroform method (Chomczynski and Sacchi, 1987). First-strand cDNA was synthesized from the A431 total RNA using oligo-dT primer and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc). The human E-cadherin cDNA was amplified by PCR from the A431 cDNA by the sense primer (5′-CGGGCTA-CCAGCCATGGGCCCTTGG-3′) containing a *Bgl*II site (underlined) and the antisense primer (5′-CGCCTCTCTA-GTCCCCTAGTGGTCC-3′) containing a *Xba*I site (underlined). PCR was performed with a Perkin Elmer Cetus Gene Amp PCR system 2400 (Norwalk, CT, USA) in a 25 μl reaction volume for 33 cycles using rTth DNA polymerase (Perkin Elmer). The PCR fragments were double digested with *Bgl*II and *Xba*I, and then ligated into a pDR2 expression vector (CLONTECH Laboratories, Inc, Palo Alto, CA, USA) (Murphy *et al*., 1992). The inserted cDNA sequences were all verified by DNA sequence analysis. The resultant plasmid, termed pDR2/E-cadherin, was transfected to U251MG cells by the liposome-mediated gene transfer method (Felgner *et al*., 1987). Briefly, 4×10^4 U251MG cells were plated onto a 10 cm dish 1 day before transfection. Eighteen micrograms of purified pDR2/E-cadherin plasmid was added to U251MG cells after a preincubation for 20 min with 28.8 μl of lipofectamine reagent and 1.5 ml of serum-free OPTI-MEN (Life Technologies, Inc). Drug selection, in 200 μg/ml Hygromycin (WAKO Pure Chemical Co, Osaka, Japan), was begun 72 h after transfection. After drug selection, colonies were harvested with cloning cylinders and expanded to cell lines.

Enzyme-linked immunosorbent assay (ELISA)

Supernatants of cultured cells treated with various reagents were filtered using a 0.22 μm millipore filter (Millipore, Bedford, MA, USA) before analysis. Soluble E-cadherin in the culture supernatant was quantified using a commercially available sandwich-type enzyme immunoassay kit (Takara Shuzo) according to the manufacturer's instructions.

Western blot analysis

For the Western blot analysis, the cultured cells were either directly lysed with sodium dodecycle sulfate (SDS) sample buffer (2% SDS, 10% glycerol, 0.1 M DTT, 120 mM Tris-HCl, pH 6.8, Bromophenol blue) or mechanically scraped followed by lysis in SDS sample buffer. Both samples were boiled for 5 min. The samples containing equal amounts of cell lysates extracted from equal numbers of cells were electrophoresed on 12% SDS-polyacrylamide gel and transferred to nitrocellulose filters with a constant current of 170 mA for 90 min. The filters were blocked in PBS containing 10% skim milk for 1 h at room temperature and then incubated with 1 : 2500 diluted anti-E-CDcyto Ab or 1 : 5000 diluted anti-β-actin Ab in PBS containing 0.03% Tween 20 for 1 h, and washed three times for 5 min each time with PBS containing 0.3% Tween 20. The filters were then incubated for 40 min with horse-radish peroxidase-conjugated anti-mouse IgG antibody (Amersham Life Science, Arlington Height, IL, USA) and, after washing with PBS containing 0.3% Tween 20, specific proteins were detected using an enhanced chemiluminescence system (Amersham Life Science).

For detection of subcellular localization of β-catenin, membrane and cytoplasmic fractions were prepared. TE13 cells treated with or without 10 μm ionomycin were suspended in five volumes of ice-cold hypotonic lysis buffer (20 mM HEPES pH 7.5, 10 mM KOAc, 1.5 mM MgOAc). After sitting on ice for 15 min, the cells were disrupted using Dounce-homogenizer. The nuclei were removed by centrifugation at 2000 *g* for 5 min at 4°C. The supernatant was centrifuged again at 10 000 *g* for 1 h at 4°C, and cytoplasmic and membrane fractions were separated. These fractions were subjected to Western blot analysis.

Preparation of anti-E-cadherin affinity column

Anti-E-CDcyto Ab was dialyzed against a coupling solution (0.1 M NaH_2PO_4/0.5 M NaCl (pH 8.3)) for 16 h at 4°C, and then bound to a 1 ml of CrBN activated agarose 4B (Pharmacia Biotech, Uppsala, Sweden) previously washed with the coupling solution. After incubation for 16 h at 4°C, the resin bound anti-E-CDcyto Ab was washed with the coupling solution and incubated with blocking buffer (1 M ethanolamine/0.2 M Na-phosphate buffer (pH 8.0)) for further 2 h at room temperature. After additional successive washing with buffer A (0.1 M acetate buffer (pH 4.0)/0.5 M NaCl) and with PBS, the resin was used for an anti-E-CDcyto affinity column. Normal mouse IgG was immobilized on another set of CrBN activated agarose column, and used for the pre-clearance of non specific binding proteins to the columns.

Purification and N-terminal amino acid sequence analysis of the 33 kD E-cadherin cleavage product

To purify the 33 kD E-cadherin cleavage product, A431 cells (50 × 10 cm culture dishes) were lysed with 25 ml of lysis buffer (PBS containing 1% NP-40, 1 mM $CaCl_2$, 1 mM AEBSF, 60 μg/ml aprotinin, 1 μM pepstatin A, 100 μM leupeptin) for 30 min at 4°C, centrifuged with 15 000 r.p.m. at 4°C. The supernatant was pre-cleared with normal mouse IgG column, and the passed through fraction was applied to the anti-E-CDcyto affinity column. The bound E-cadherin cleavage product to the column was eluted with 0.2 M glycin buffer (pH 2.8), dialyzed against distilled water, and concentrated with vacuum concentrator. After direct separation by SDS–PAGE, the E-cadherin cleavage product was transferred on to a polyvinylidene difluoride (PVDF) membrane. The 33 kD protein band corresponding to the E-cadherin cleavage product on the PVDF membrane was stained with Coomassie brilliant blue, and its N-terminal amino acid sequence was analysed by the Procise 492 Protein sequencer system (Perkin Elmer Applied Biosystems).

In vitro *E-cadherin cleavage reaction*

Membrane fractions of U251/E-cadherin cells were prepared as described above. The sedimented membranes were added to ten volumes of ice-cold 1 M NaCl in 30 mM Tris-HCl (pH 7.2) and kept 15 min on ice. Membranes were collected by centrifugation at 10 000 *g* for 15 min at 4°C. Washed membranes were reconstituted in ice-cold Tris-HCl (30 mM, pH 7.2). The membrane fraction with or without BB2516 was incubated for 120 min at 37°C. The reaction was stopped by the addition of an equal quantity of SDS sample buffer and incubated at 100°C for 5 min. Then, aliquots of reaction mixtures were subjected to Western blot analysis using anti-E-CDcyto Ab.

Immunofluorescence microscopic analysis

TE13 cells grown on Lab-Tek II Chamber Slide (Nalge Nunc Int, Naperville, IL, USA) were treated with various components to be tested. Then the cells were fixed with 4% paraformaldehyde for 10 min followed by 0.2% Triton X-100 in PBS for 5 min. After being washed with PBS, the cells were incubated with HECD-1 Ab, anti-E-CDcyto Ab or anti-β-catenin Ab diluted in PBS containing 0.2% bovine serum albumin (BSA) for 60 min at room temperature, washed three times in PBS, and then incubated for 60 min at room temperature with FITC-conjugated anti-mouse Ab (Biosource, Camarillo, CA, USA) for 60 min. After being washed with PBS, samples were mounted in 80% glycerol and visualized with a confocal microscope (Fluoview, Olympus, Tokyo, Japan) equipped with an argon gas laser and appropriate filter sets to allow the stimultaneous recording of fluorescein. Fluorescence micrographs were recorded using PLAPO 40 × objectives and were sampled at 1024 × 1024 pixels and 8-bit resolution per color.

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