

Expression of α -catenin in α -catenin-deficient cells increases resistance to sphingosine-induced apoptosis

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α -Catenin, an intracellular protein, associates with the COOH-terminal region of cadherin cell adhesion molecules through interactions with either β -catenin or γ -catenin (plakoglobin). The full activity of cadherins requires a linkage to the actin cytoskeleton mediated by catenins. We transfected α -catenin-deficient colon carcinoma cells with a series of α -catenin constructs to determine that α -catenin expression increases the resistance to apoptosis induced by sphingosine. Two groups of constructs, containing deletions in either the middle segment of the molecule or the COOH terminus, induced morphological changes, cell compaction, and decreases in cell death. In α -catenin-expressing cells, inhibition of cadherin cell adhesion by treatment with anti-E-cadherin antibodies

did not decrease the cells viability. α -Catenin expression partially suppressed the downregulation of Bcl-xL and the activation of caspase 3. Expression of p27kip1 protein, an inhibitor of cyclin-dependent kinases, was increased by α -catenin expression in low density cell cultures. The increased levels of p27kip1 correlated with both increased resistance to cell death and morphological changes in transfectants containing deletion mutants. Transfection-mediated upregulation of p27kip1 decreases sphingosine-induced cell death in α -catenin-deficient cells. We postulate that α -catenin mediates transduction of signals from the cadherin-catenin complex to regulate the apoptotic cascade via p27kip1.

Introduction

The cadherin family of transmembrane glycoproteins plays an essential role in the initiation and stabilization of cell-cell contacts (Takeichi, 1991; Kemler, 1993; Gumbiner, 1996; Marrs and Nelson, 1996). A conserved cytoplasmic domain, common to these proteins, interacts with intracellular proteins termed catenins (Ozawa et al., 1989, 1990; Ozawa and Kemler, 1992; Stappert and Kemler, 1994). The extracellular domain is responsible for specific homophilic binding (Nose et al., 1990). Classical cadherins, including E-cadherin, bind to either β -catenin or γ -catenin (plakoglobin), which links this complex to α -catenin. α -Catenin, a 102-kD protein, contains multiple interaction sites: actin-binding sites (Rimm et al., 1995), binding sites for other actin-binding proteins such as α -actinin (Nieset et al., 1997), vinculin (Watabe-Uchida et al., 1998), and ZO-1 (Itoh et al., 1997; Imamura et al., 1999), and homodimerization sites (Koslov et al., 1997). Without α -catenin, cells do not associate tightly with each other despite the expression of cadherins (Watabe et al., 1994; Ozawa, 1998; Maeno et al., 1999).

Therefore, the interactions of α -catenin, linking the cadherin-catenin complexes to the actin cytoskeleton, may be essential to mediate the full activity of cadherins.

Cadherin molecules on the cell surface transduce extracellular signals (Takeichi, 1991; Larue et al., 1996), possibly altering cell polarity (McNeill et al., 1990; Watabe et al., 1994), growth rate (Watabe et al., 1994; Bullions et al., 1997), and cell-substratum adhesion (Miyaki et al., 1995). However, the mechanism by which cadherin-catenin complexes regulate cell fate remains to be investigated.

To examine the functions of α -catenin, we established DLD-1/ $\Delta\alpha$ cell clones transfected with α -catenin (Ozawa, 1998). DLD-1/ $\Delta\alpha$ is derived from the DLD-1 human colon cancer cell line, lacking endogenous expression of α -catenin. Cadherin-mediated cell adhesion is disrupted in this variant, despite the presence of other cadherin cell adhesion complex components: E-cadherin, β -catenin, and γ -catenin. We searched for the functions of α -catenin in signal transduction and found a significant reduction in death of α -catenin-expressing clones after treatment with sphingosine, an inducer (Sakakura et al., 1996; Sweeney et al., 1996; Shirahama et al., 1997) and endogenous mediator (Ohta et al., 1994, 1995) of apoptosis. The basic molecular framework for regulating and executing apoptosis comprises a functionally ordered product of expanded gene families, including

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the caspases and Bcl-2 family of proteins. The cadherin/catenin-derived signal may affect the functions and activities of the molecules in the apoptotic pathways. Therefore, examination of the effects of cadherin signaling on death mediators and/or regulators may uncover the molecular mechanism underlying the signal.

To determine the region of the molecule responsible for reductions in cell death induction, we examined the cellular phenotypes, resistance to cell death, and cell morphology, resulting from deletions in α -catenin. We compared the effects of anti-E-cadherin antibody treatment with α -catenin deficiency. We then analyzed the status of death mediator molecules in the apoptosis cascade to find possible mediators of the signals from cadherin–catenin complexes. Our results indicate that increases in p27kip1, an inhibitor of cyclin-dependent kinases (cdks), correlates with resistance to cell death in the α -catenin transfectants, suggesting that expression of α -catenin effects the cell death through the anti-apoptotic function of p27kip1. Transfection-mediated upregulation of p27kip1 levels in α -catenin–deficient cells decreases the levels of cell death induced by sphingosine, supporting our hypothesis.

Results

Reduction of sphingosine-induced cell death by α -catenin expression

α D cells, a stable transfectant of a DLD-1/ $\Delta\alpha$ variant, express full-length human α -catenin; nD cells are a control vector transfectant (Ozawa, 1998). To induce apoptosis, cells were plated at 5×10^4 cells per 35-mm dish for 24 h and then treated with 5–25 μ M sphingosine. Cell viability was then assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)* assay. α D cells demonstrated significant resistance to sphingosine-induced cell death compared with nD cells (Fig. 1 A). When cells were treated with 25 μ M sphingosine, a relatively high concentration, almost all of the cells died within 7 h; there was no detectable difference between α D and nD cells. Upon treatment with lower concentrations of 15–17.5 μ M, cells died in a time-dependent manner; within 18–24 h, 50–80% of nD cells had been killed. At these critical concentrations of sphingosine, α D cells survived with reduced levels of cell death. To confirm that observed cell death occurs through apoptosis, we extracted low molecular weight fragmented DNA from the cells and analyzed the fragments by electrophoresis. Sphingosine induced significant degradation of DNA into oligo-nucleosomal size fragments in nD cells (Fig. 1 B), a hallmark of apoptosis. In addition, direct measurement of nuclear morphology, staining with DAPI, visualized condensed chromatin and fragmented nuclei (Fig. 1 C), another characteristic of apoptosis. Therefore induction of apoptosis after the experimental treatment with sphingosine was supported by multiple methods.

*Abbreviations used in this paper: Ac-DEVD-cmk, acetyl-Asp-Glu-Val-Asp-chloromethylketone; Ac-DEVD-pNA, acetyl-Asp-Glu-Val-Asp-p-nitro-anilide; cdk, cyclin-dependent kinase; mAb, monoclonal antibody; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

In high density cultures, cell death is not efficiently induced regardless of α -catenin expression (unpublished data), making cell density an important factor in this phenomenon. When treated with sphingosine in the absence of FCS, cells died as quickly as cells treated with high concentrations of sphingosine in the presence of FCS. As a result, no differences in viability were observed between α D cells and nD cells in the absence of FCS (unpublished data).

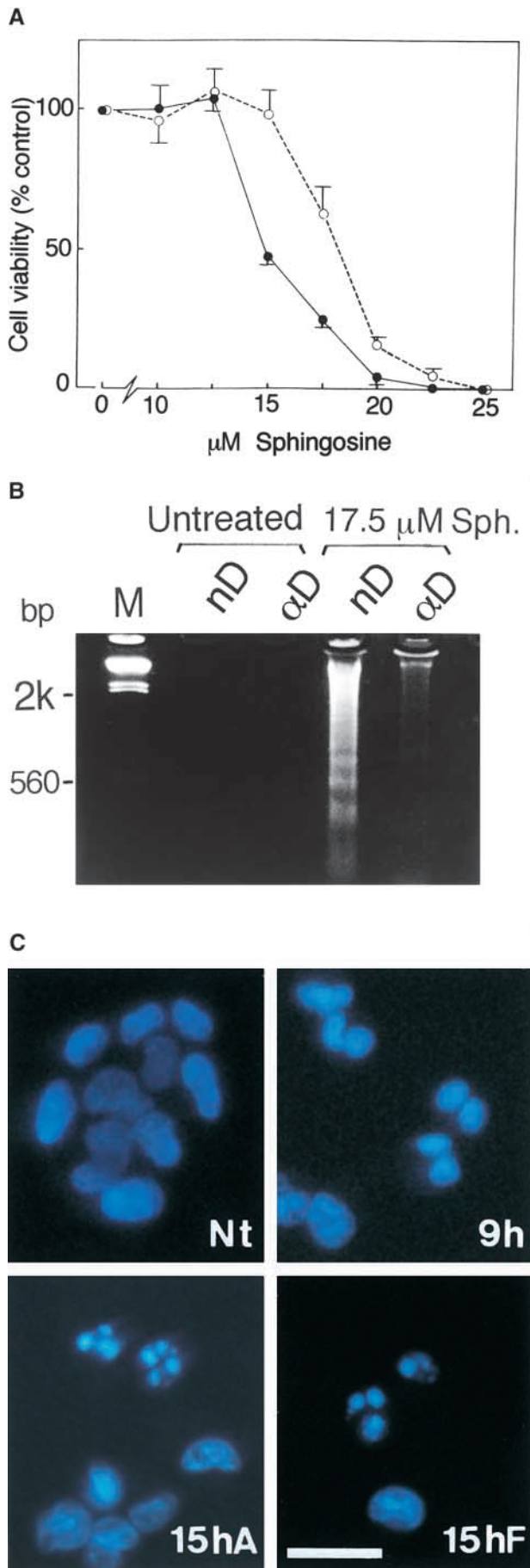
Therefore, α -catenin expression in DLD-1/ $\Delta\alpha$ cells reduced sphingosine-mediated induction of cell death. At the critical concentrations of the inducer, this effect was observed in low density cultures in the presence of FCS. The effect was significant and reproducible.

To ascertain the role of α -catenin in the reduction of sphingosine-induced cell death, we analyzed pairs of cells containing or lacking α -catenin. PC 3, a prostate cancer cell line, lacks α -catenin due to homozygous deletion of the gene (Morton et al., 1993). We transfected PC 3 cells with the construct used to transfect the DLD-1/ $\Delta\alpha$ cells to establish clones expressing α -catenin (Fig. 2 A). With these cells, we got similar results (Fig. 2 B). Upon treatment with sphingosine, we observed a similar reduction of cell death dependent on α -catenin. The results indicate that the reduction in levels of sphingosine-induced cell death is a general cellular phenotype, resulting from the expression of α -catenin.

Identification of the region of α -catenin that confers the increased resistance to cell death

To identify the region of α -catenin necessary to mediate the anti-cell death effect, we created stable transfectants expressing a series of α -catenin deletion mutants (Fig. 3 A). These cells expressed α -catenin polypeptides, containing the NH₂-terminal β -/ γ -catenin-binding site and either the middle or COOH-terminal region. Immunoblot analysis demonstrated that each transfectant expressed proteins of the expected size; the levels of expression did not fluctuate significantly (Fig. 3 B).

Deletion of amino acids 203–611 in the middle segment of the protein ($\alpha\Delta$ NM1D cells) or of amino acids 614–906 in the COOH-terminal region of α -catenin ($\alpha\Delta$ C1D cells) did not reduce the viability of transfectants after treatment with sphingosine (Fig. 3 C). External deletion of the α -catenin COOH terminus demonstrates that amino acids 1–381 are sufficient to mediate the increase of viability. In contrast, further deletion of amino acids 203–688 ($\alpha\Delta$ NM2) or external deletion of amino acids 303–906 ($\alpha\Delta$ C6) reduced the viability of transfectants compared with cells transfected with either full-length α -catenin transfected (α D) or the deletion mutants, $\alpha\Delta$ NM1D, $\alpha\Delta$ C1D, and $\alpha\Delta$ C4D. This data indicated that both the middle and COOH-terminal regions of α -catenin are dispensable for this anti-cell death effect. These regions function similarly, acting redundantly to reduce cell death. In other words, the combination of the NH₂-terminal β -/ γ -catenin-binding site (amino acids 48–163 in the used fragment of amino acids 1–202; Obama and Ozawa, 1997) with either amino acids 203–381, in the middle region of the molecule, or amino acids 612–906, at the COOH terminus, can induce an increased resistance of transfectants to sphingosine-induced cell death compared with α -catenin–deficient cells.



All transfectants exhibiting resistance to cell death demonstrated compacted aggregation in multicellular suspension cultures; transfectants sensitive to cell death were loosely adherent, existing as “grape-like” aggregates in multicellular suspension cultures (Fig. 3 D). Cell compaction (Kemler et al., 1977; Takeichi, 1977; Hyafil et al., 1980) results from a cellular morphological change in which cells adhere more tightly to each other, maximizing contact areas. This effect is observed in association with cadherin-mediated cell–cell adhesion (Vestweber and Kemler, 1985), involving forces generated by the actin cytoskeleton (Surani et al., 1990). Therefore, cells demonstrating the compacted morphotype suggest a link between the cadherin–catenin complex and the actin cytoskeleton. These data suggest that transfectants expressing α -catenin variants able to associate with the actin cytoskeleton, inducing compaction in three-dimensional cultures, are resistant to spingosine-induced cell death.

α D, $\alpha\Delta$ C1D, and $\alpha\Delta$ C4D cells demonstrated an epithelioid morphotype when plated on solid substrate; $\alpha\Delta$ NM2D, $\alpha\Delta$ C6D, and nD cells showed a nonepithelioid morphotype with highly refractile cell borders. When plated on plastic, $\alpha\Delta$ NM1D cells exhibit refractile borders but not to the same extent as $\alpha\Delta$ NM2D, $\alpha\Delta$ C6D, and nD cells. It remains unclear why $\alpha\Delta$ NM1D cells do not exhibit an epithelial morphology on solid substrates, although these cells compact in multicellular suspension cultures. Cell–substratum adhesion may weaken the cell–cell adhesive interactions of DLD-1 cells.

Anti-E-cadherin antibodies induce disruption of epithelioid morphotype but do not sensitize α D cells to spingosine-induced cell death

To examine the relationship of this change in morphology to the α -catenin–dependent resistance to spingosine-induced cell death, we used an anti-human E-cadherin antibody, SHE78-7, to disrupt the cell–cell contacts of α D cells. The addition of SHE78-7, a function-blocking monoclonal

Figure 1. Spingosine induces apoptosis in DLD-1/ $\Delta\alpha$ cells, and expression of α -catenin decreases levels of spingosine-induced cell death. (A) α -Catenin (α D cells, \circ) and control (nD cells, \bullet) transfectants of DLD-1/ $\Delta\alpha$ cells were treated with the indicated concentrations of spingosine for 18 h. Cell viability was determined by the MTT assay, indicated here as a percentage of the absorbance observed in the absence of spingosine. The indicated results are the means and SD of triplicate values obtained in one representative experiment of five. (B) Spingosine (Sph)-induced internucleosomal DNA fragmentation in nD and α D cells. Cells were treated with 17.5 μ M spingosine for 18 h. The fragmented DNA was then extracted, analyzed as described in the Materials and methods, and visualized on a 2% agarose gel. The method does not extract intact DNA from normal cells. (C) Spingosine-induced nuclear condensation and fragmentation. nD cells were treated with 17.5 μ M spingosine for 9 or 15 h and then stained with DAPI as described in the Materials and methods. Floating cells were recovered by centrifuge and also stained. Ut, cells not treated with spingosine; 9H, attached cells treated with spingosine for 9 h; 15hA, attached cells treated for 15 h; 15hF, floating cells treated for 15 h. Essentially the same patterns of nuclear morphology were also detected in α D cells, both in attached and floating cells after spingosine treatment, although the total number of floating cells were reduced in α D cells. Bar, 20 μ m.

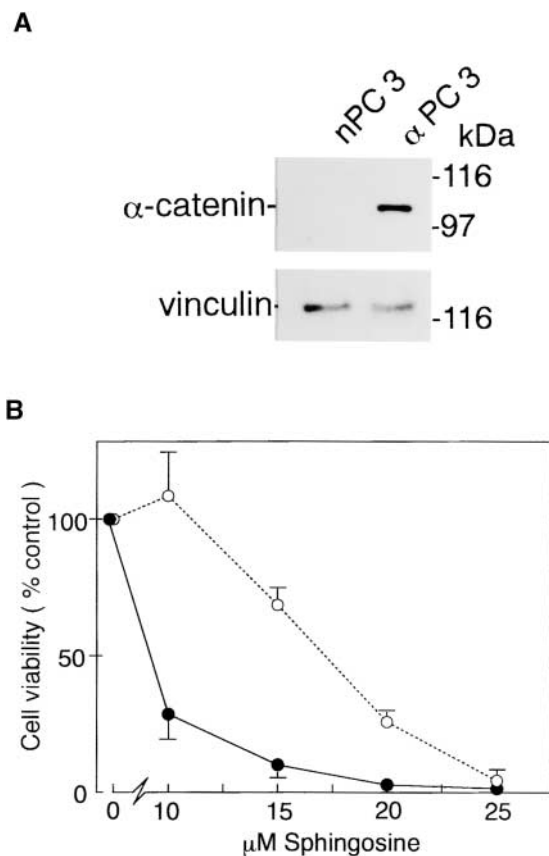


Figure 2. Expression of α -catenin decreases sphingosine-induced cell death of PC 3 cells. (A) Immunoblot detection of the α -catenin polypeptide in PC 3 transfectants. (B) α -Catenin (α PC 3 cells, \circ) and control (n PC 3 cells, \bullet) transfectants of PC 3 cells were treated with the indicated concentrations of sphingosine for 18 h. Cell viability was determined by the MTT assay, indicated here as a percentage of absorbance observed in the absence of sphingosine. The indicated results are the means and SD of triplicate values obtained in one representative experiment of three.

antibody (mAb), to cultures disrupts cell–cell contacts to induce decompaction (Watabe et al., 1994; Kantak and Kramer, 1998; St. Croix et al., 1998). In the presence of this antibody, α D cells demonstrated a nonepithelioid morphology on solid substrates, similar to that seen with nD cells (Fig. 4 A). Concentrations from 3 to 100 μ g/ml induced a similar morphology without any observable toxicity. Treatment with sphingosine did not reduce the viability of α D cells in the presence of anti-E-cadherin antibodies (Fig. 4 B). Both the MTT assay and direct counting of cells after trypsin treatment confirmed that no difference in levels of cell death was observed between antibody-treated and untreated α D cells. SHE78-7 antibodies induce disruption of homophilic cadherin binding; they are not thought to signal

as a cadherin ligand. Treatment of PC-9 cells, an α -catenin-deficient cell line, with this antibody overcomes the effect of α -catenin expression, resulting in cell dissociation and growth stimulation (Watabe et al., 1994). These results indicate that the inhibition of cell–cell adhesion by function-blocking antibodies does not have the same effect as the inhibition caused by a deficiency of α -catenin, although the cell morphotype induced by both types of inhibition is quite similar. Therefore, close cell–cell contacts are not required for resistance to cell death.

The Bcl-xL protein level in nD cells is lower than that in α D cells during induction of cell death by sphingosine

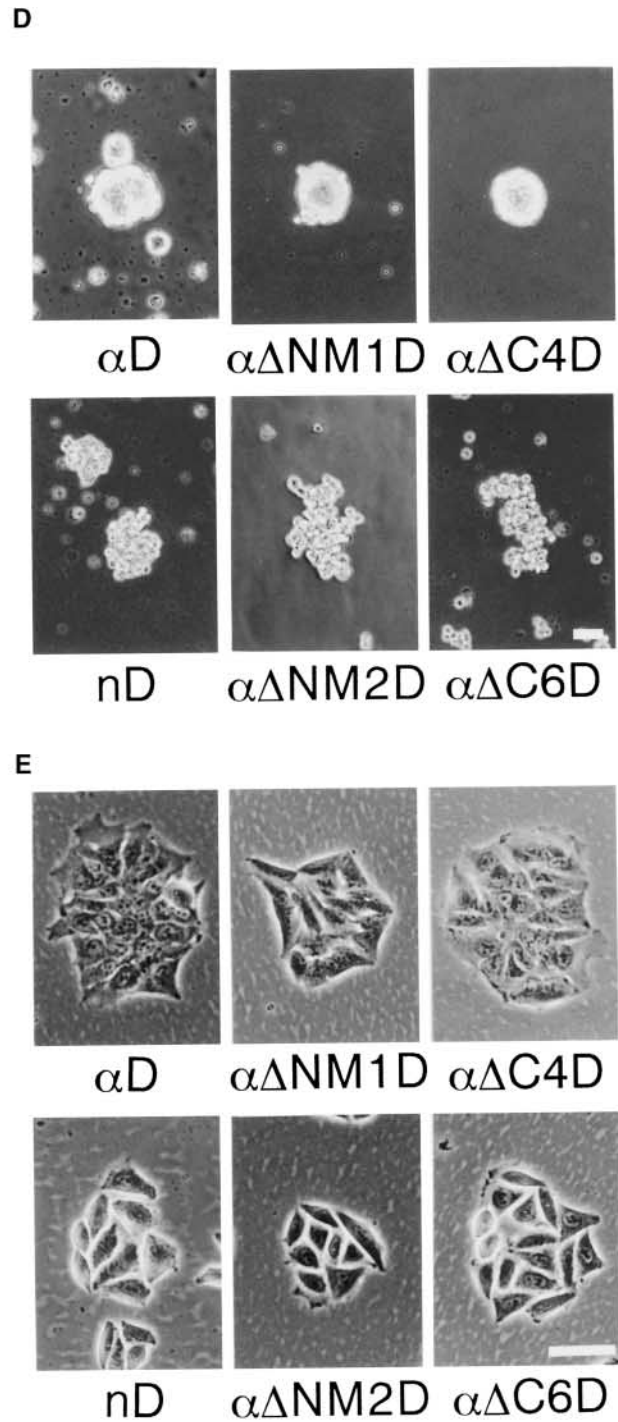
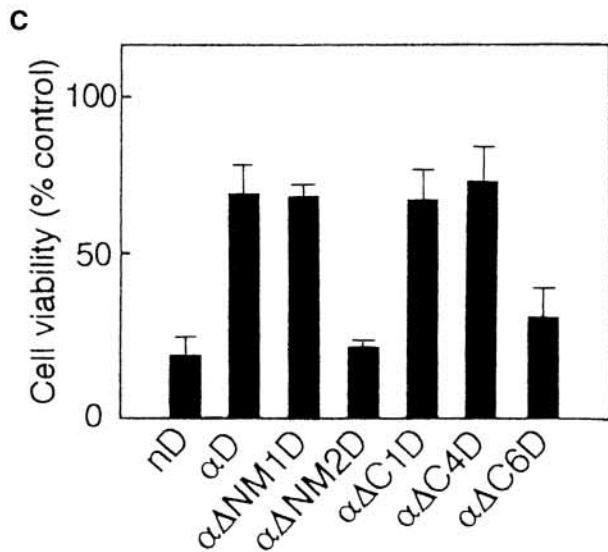
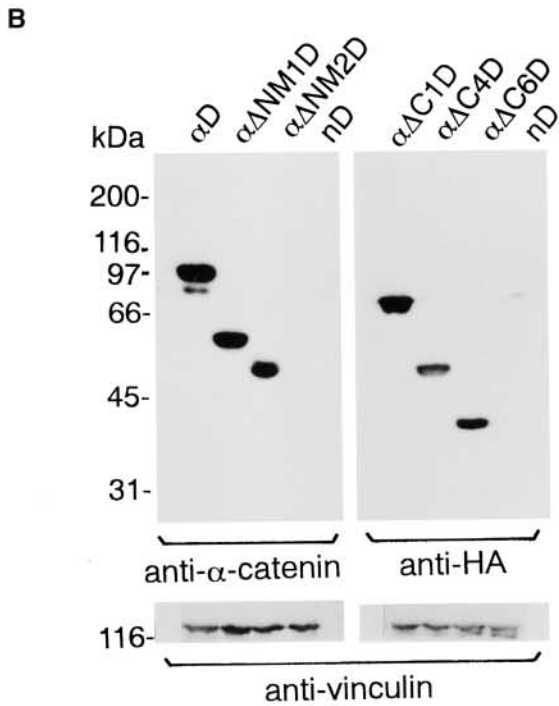
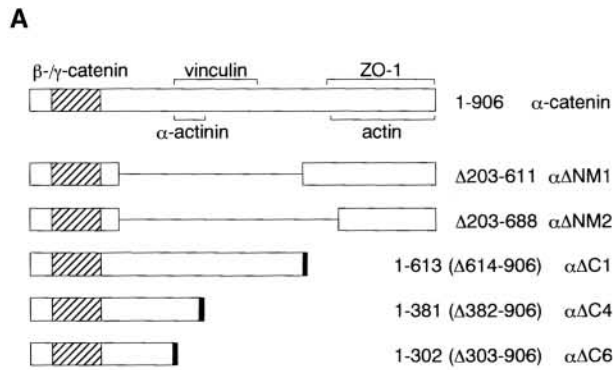
The Bcl-2 family of proteins plays a central role in the regulation of apoptotic cell death. Overexpression of the antiapoptotic members of this family, Bcl-2 and Bcl-xL, inhibits apoptosis induced by a wide variety of stimuli (Cory, 1995; Korsmeyer, 1995; Reed, 1997). Sphingosine-induced apoptosis was accompanied by a concomitant decrease on Bcl-2 or Bcl-xL expression as exhibited on both the RNA and protein levels (Sakakura et al., 1996; Shirahama et al., 1997).

We detected Bcl-xL protein but not Bcl-2 protein in both α D cells and nD cells by Western blotting (unpublished data). Sphingosine treatment downregulated the total cellular levels of Bcl-xL protein; this decrease occurred more quickly in nD cells than in α D cells. After a 9–12-h treatment with sphingosine, Bcl-xL levels in α D cells were greater than those observed in nD cells as observed by Western blot analysis of equivalent protein amounts (Fig. 5 A). Differences in Bcl-xL levels were also detected at 3–6 h, before the cells demonstrated the morphological changes that accompany apoptosis. After a 15–24-h treatment with sphingosine, levels of Bcl-xL and vinculin both decreased in nD cells. Since a large fraction of nD cells floated and died in this period, these differences in protein levels could result from cell death. In contrast, no differences in the levels of vinculin were detected between α D and nD cells until 12 h after treatment. These data suggest that at least one target mediating the increased resistance of α D cells to cell death may exist upstream of Bcl-xL.

Activation of terminal caspase (caspase 3-like proteases) is partially suppressed in α D cells

The caspase family of proteins, highly specialized cysteine proteases, is essential in the regulation and implementation of apoptosis (Alnemri, 1997; Salvesen and Dixit, 1997; Núñez et al., 1998). Initiator caspases are activated by apoptotic stimuli to activate downstream effector caspases by proteolytic cleavage in an amplifying protease cascade. Caspase 3 (CPP 32/Yama) is a key effector caspase, cleaving substrates after a DEXD sequence. Z-DEVD-fmk, an inhibitor of caspases 3 and 7, and the wide spectrum caspase inhibitor, Z-VAD-fmk,

Figure 3. Transfectants expressing mutant α -catenins, inducing compaction in three-dimensional culture, demonstrate reduced sensitivity to sphingosine-induced cell death. (A) Schematic representation of α -catenin (top) and mutant α -catenin polypeptides with internal or COOH-terminal deletions. The black box designates a HA tag. (B) Immunoblot detection of the α -catenin polypeptide in DLD-1/ α cells expressing either full-length or mutant α -catenins. Full-length (α D cells) and mutant α -catenin polypeptides possessing deletions in the middle segment of the molecule ($\alpha\Delta$ NM1D and $\alpha\Delta$ NM2D cells) were detected with an anti- α -catenin mAb; mutant α -catenin polypeptides with COOH-terminal deletions ($\alpha\Delta$ C1D, $\alpha\Delta$ C4D, and $\alpha\Delta$ C6D cells) were detected using an anti-HA mAb. (C) Cell viability of stable transfectants expressing mutant α -catenins. After treatment for 18 h with 17.5 μ M sphingosine, cell viability was determined as in the legend to Fig. 1. The results are the means and SD of the values obtained in three independent experiments. (D) Aggregates of transfectants expressing mutant



α -catenins in multicellular suspension cultures. Cells were seeded in 35-mm dishes, coated previously with polyhydroxyethylmethacrylate, at 5×10^4 cells per dish and then incubated for 2 d. (E) Morphology of transfectants expressing mutant α -catenins when plated on a solid substrate. The cell-cell contacts of nD, $\alpha\Delta$ NM2D, and $\alpha\Delta$ C6D cells but not of α D, $\alpha\Delta$ NM1D, and $\alpha\Delta$ C4D cells is clear. nD, $\alpha\Delta$ NM2D, and $\alpha\Delta$ C6D cells exhibit highly refractile borders, different from the borders of α D, $\alpha\Delta$ NM1D, and $\alpha\Delta$ C4D cells. Bars, 50 μ m.

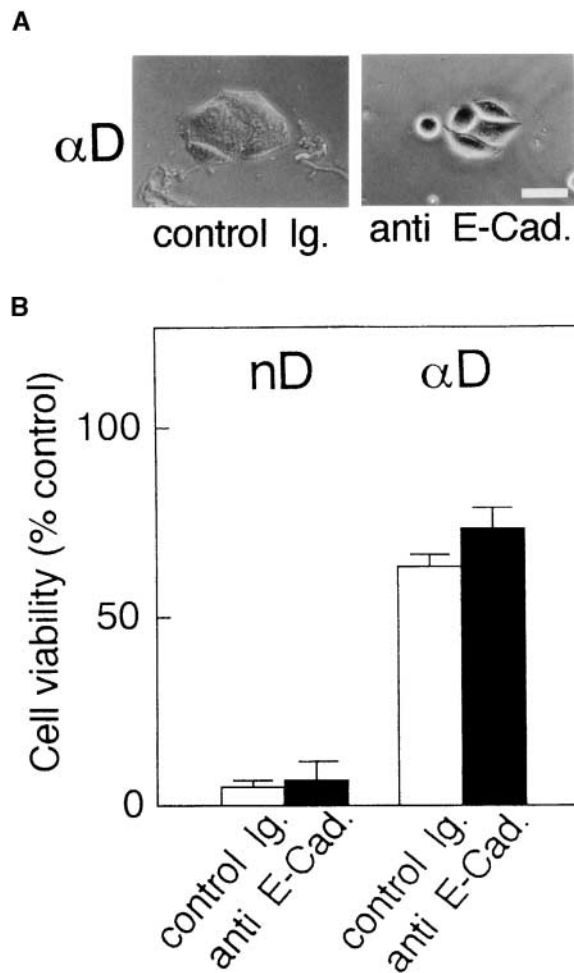


Figure 4. Anti-E-cadherin-blocking antibodies disrupt the epithelioid morphology but do not influence sphingosine-induced cell death in α D cells. (A) Morphological changes of α D cells after treatment with anti-E-cadherin-blocking antibodies. α D cells were cultured in either 100 μ g/ml of SHE78-7 antibodies or control mouse IgG for 1 d. (B) nD and α D cells were cultured in either 100 μ g/ml of SHE78-7 antibodies or control mouse IgG for 1 d, then treated with 17.5 μ M sphingosine for 18 h in the continuing presence of the antibody. Cell viability was determined by the MTT assay. The results are shown as the means and SD of the values obtained in four independent experiments. Bar, 50 μ m.

inhibit sphingosine-induced apoptosis (Sweeney et al., 1998), suggesting that the target of sphingosine exists upstream of caspase 3 (or caspase 3-like proteases). We used a tetrapeptide substrate to measure caspase 3 (-like) activity in cells after treatment with sphingosine. Activity was elevated in both nD and α D cells after treatment for 9 h with sphingosine; this elevation was reduced in α D cells compared with nD cells (Fig. 6). This result indicated that the expression of α -catenin could inhibit the activation of an effector caspase; the target(s) of the signal from α -catenin may intervene with the death signal upstream of caspase 3 (or caspase 3-like proteases).

p27kip1 protein in α D cells is upregulated in low density cultures

E-cadherin-dependent growth suppression in three-dimensional cultures is mediated by increases in p27kip1, a cdk inhibitor (St. Croix et al., 1998). Upregulation of p27kip1 is

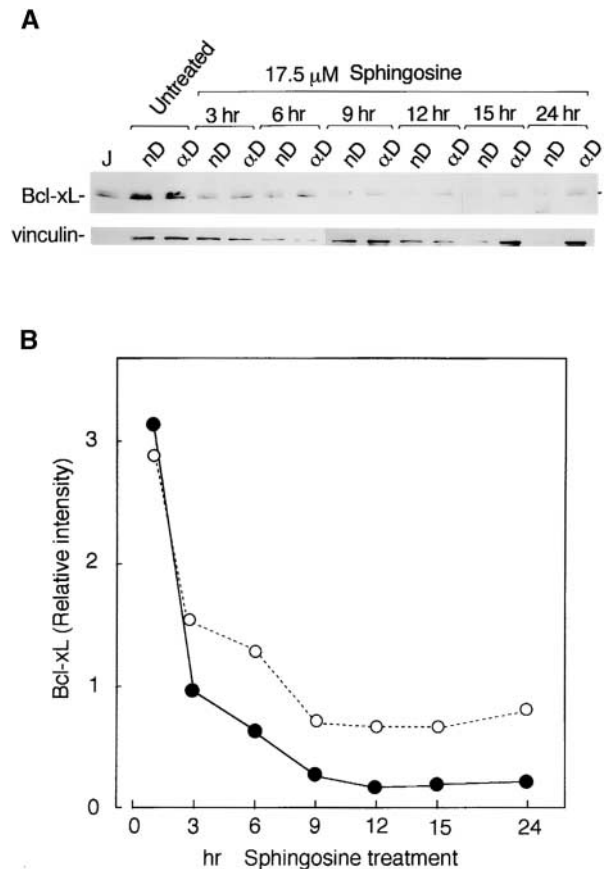


Figure 5. Bcl-xL protein levels in nD and α D cells during sphingosine induction of cell death. (A) Cells were treated with 17.5 μ M sphingosine for the indicated times. 15 μ g of protein per lane obtained from whole cell extracts was subjected to Western blot analysis using antibodies against Bcl-xL and vinculin. Antivinculin antibodies were used to identify fluctuations in the protein amount between nD and α D cells. J indicates Jurkat cells, used as a positive control for Bcl-xL protein. The experiment was repeated four times with similar results obtained each time. (B) The relative intensity of Bcl-xL bands in A was determined by densitometry. These values are indicated as the ratio to the intensity of Jurkat cells (= 1.0). ●, nD cells; ○, α D cells.

also involved in N-cadherin-mediated contact inhibition of cell growth and entry into S phase (Levenberg et al., 1999). In addition, p27kip1 provides resistance to cytotoxic drugs and reduces apoptosis in carcinoma cells grown in confluent monolayers (Dimanche-Boitrel et al., 1998), multicellular spheroids (St. Croix et al., 1996), and leukemia cells (Eymin et al., 1999a). To examine the role of this cdk inhibitor in resistance to sphingosine-induced cell death, we examined the levels of p27kip1 in nD and α D cells.

In confluent cultures, p27kip1 protein levels were high; no differences were detectable between nD and α D cells (Fig. 7). After trypsin/EDTA treatment, p27kip1 protein levels decreased significantly (unpublished data). After plating 2×10^5 cells per 100-mm dish, p27kip1 protein levels gradually increased during a 3-d incubation. In these low density cultures, higher levels of p27kip1 were observed in α D cells in comparison to nD cells (Fig. 7). Cell death was not efficiently induced in high density cultures, either confluent cells or cells cultured for more than 3 d after seeding as

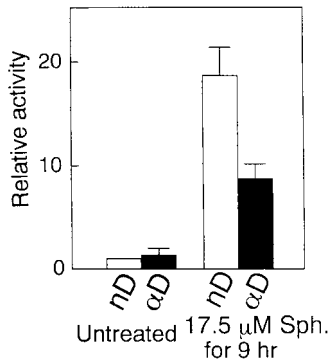


Figure 6. **The activation of caspase 3 (–like proteases) is partially suppressed in α D cells.** After treatment with 17.5 μ M sphingosine (Sph) for 9 h, cell extracts were used to determine the caspase 3 (–like) activity with Ac-DEVD-pNA as a substrate. Relative activities are indicated as a fold increase as compared with the activity of untreated nD cells (= 1.0). The results are shown as the means and SD of the values obtained in three independent experiments.

above, regardless of α -catenin expression (unpublished data). These results suggest a correlation between p27kip1 expression and resistance to cell death. DLD-1/ $\Delta\alpha$ transfectants expressing a level of p27kip1 above a certain threshold, such as that seen in high density cultures, cannot be induced to undergo cell death by sphingosine treatment. Cells expressing lower levels of p27kip1, such as cells present in low density cultures, undergo cell death after sphingosine treatment. In this case, the upregulation of p27kip1 resulting from α -catenin expression correlates with reductions in cell death.

The correlation between increased levels of p27kip1 and increased resistance to cell death in low density cultures was also observed for transfectants expressing α -catenin deletion mutants. The α -catenin mutant transfectants, $\alpha\Delta$ NM1D, $\alpha\Delta$ C1D, and $\alpha\Delta$ C4D cells, demonstrated an upregulation in the levels of p27kip1 similar to those of α D cells in low density cultures. In contrast, the expression of p27kip1 in $\alpha\Delta$ NM2D and $\alpha\Delta$ C6D cells was reduced, similar to levels seen in nD cells (Fig. 8). p21cip1, another member of the CIP/KIP family of kinase inhibitors, was not detectable by Western blot analysis in either nD or α D cells (unpublished data).

Increased expression of p27kip1 reduces sphingosine-induced cell death of α -catenin-deficient (DLD-1/ $\Delta\alpha$) cells

To assess the role of p27kip1 in resistance to sphingosine-induced cell death, we overexpressed p27kip1 in α -catenin-deficient (DLD-1/ $\Delta\alpha$) cells. Cells were stably transfected with a vector containing the full-length human p27kip1 cDNA with an HA tag. We selected clones by Western blot analysis using anti-p27kip1 antibodies (Fig. 9 A). Quantitative immunoblotting, after making serial dilutions of extracts, revealed that representative clones (p27HAD cells) contained a total amount of p27kip1 protein (tagged and endogenous protein) approximately two to three times that of control transfectants in low density cultures, the conditions used in sphingosine treatment (unpublished data). This is a similar amount to α D cells in the same conditions (Figs. 7 and 8).

Elevated expression of p27kip1 in DLD-1/ $\Delta\alpha$ cells re-

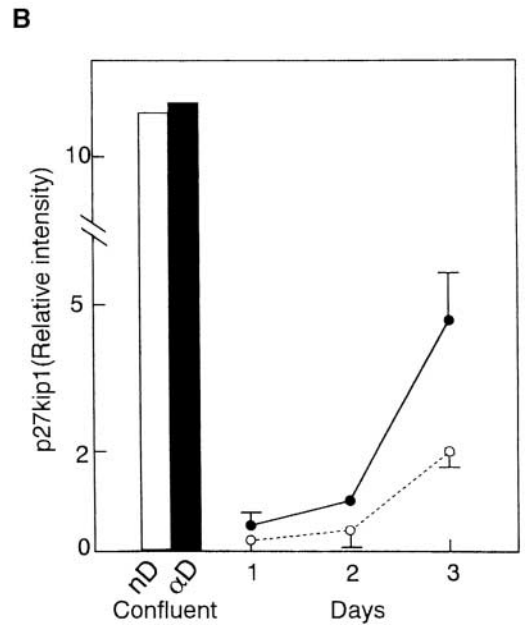
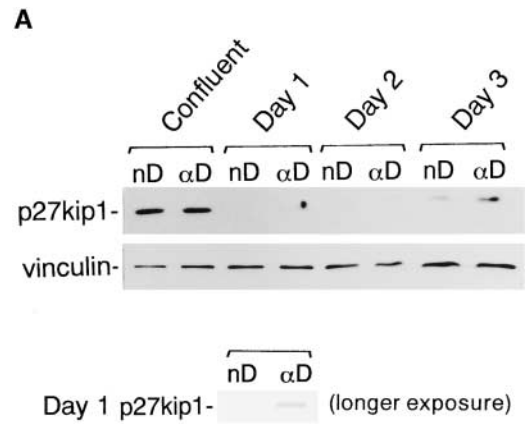


Figure 7. **Levels of p27kip1 are upregulated in low density cultures of α D cells.** (A) Cells were grown to confluency and then treated with trypsin/EDTA. Single cell suspensions were seeded at 2×10^5 cells per 100-mm dish, then incubated for the indicated times. 20 μ g of protein per lane, obtained from whole cell extracts, was subjected to Western blot analysis using antibodies against p27kip1 and vinculin. Antivinculin antibodies were used to identify fluctuations in total protein amounts. A longer exposure of the blot (Day 1) is shown at the bottom. (B) The relative intensity of p27kip1 was measured by densitometry and calculated from three independent experiments. Values are shown as the ratio to the intensity of α D cells on day 2 (= 1.0). \circ , nD cells; \bullet , α D cells. The p27kip1 protein levels in confluent nD and α D cells are represented as hatched and black bars, respectively.

duced the level of cell death induced by sphingosine (Fig. 9 B). This resistance to cell death was observed with the critical inducer concentrations observed for nD and α D cells. These results suggest that increased p27kip1 levels mediate the increased survival of α -catenin-expressing cells.

α -Catenin expression does not suppress the growth of DLD-1/ $\Delta\alpha$ cells

p27kip1 inhibits cyclin E-CDK 2 activity implicated in the negative regulation of G1 progression. Therefore, we exam-

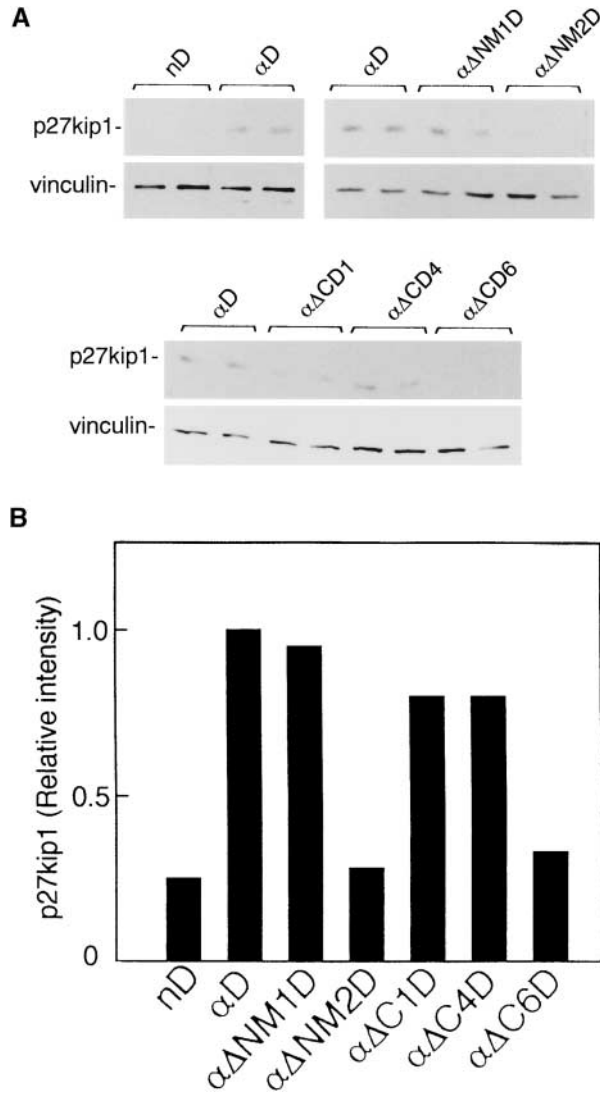


Figure 8. Mutant α -catenin transfectants exhibiting close cell-cell contacts demonstrate increased levels of p27kip1 in low density cultures. (A) Cells were seeded at 2×10^5 cells per 100-mm dish and incubated for 2 d. 25 μ g of protein per lane, obtained from whole cell extracts, was subjected to Western blot analysis using antibodies against p27kip1 and vinculin. Antivinculin antibodies identified fluctuations in total protein levels. (B) The relative intensity of p27kip1 bands was measured by densitometry. Values were calculated from two independent experiments as the ratio to the intensity of α D cells on day 2 (= 1.0).

ined the effect of p27kip1 upregulation in low density cultures of α D cells on cell growth. α -Catenin expression did not affect the growth of DLD-1/ $\Delta\alpha$ cells (Fig. 10), in contrast to previous results with PC 9 cells (Watabe et al., 1994) and Ov2008 cells (Bullions et al., 1997). The growth of these two α -catenin-deficient cell lines is retarded after the restoration of α -catenin expression.

Discussion

The exogenous expression of α -catenin in α -catenin-deficient cells restores and/or enhances cell adhesion to result in a compacted and/or an epithelioid morphotype (Watabe et al., 1994; Ewing et al., 1995; Bullions et al., 1997; Ozawa, 1998;

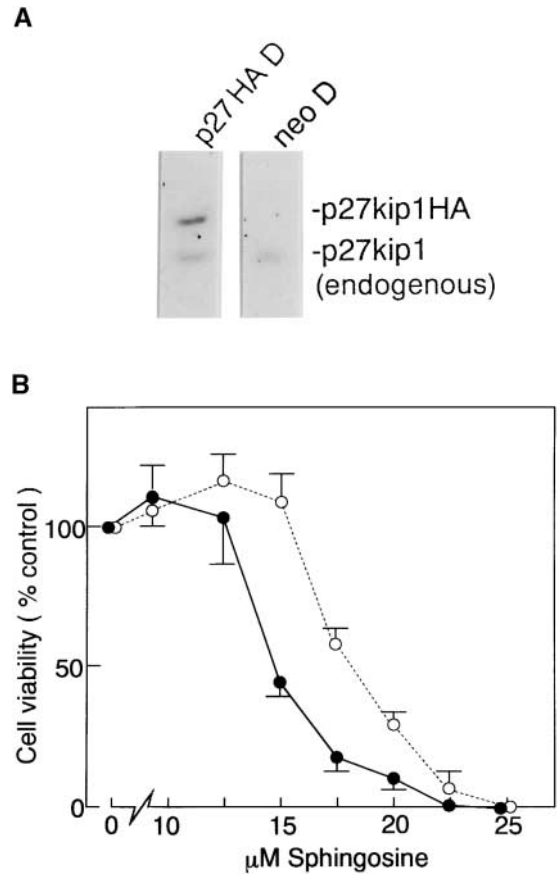


Figure 9. Transfection-mediated upregulation of p27kip1 levels in α -catenin-deficient cells decreases levels of sphingosine-induced cell death. (A) Immunoblot detection of p27kip1 protein in p27HA and control transfectants of DLD-1/ $\Delta\alpha$ cells. 25 μ g of protein per lane, obtained from whole cell extracts, was subjected to Western blot analysis using antibodies against p27kip1. (B) DLD-1/ $\Delta\alpha$ cells expressing exogenous p27 (p27HAD cells, ○) and control transfectants (neoD cells, ●) were treated for 18 h with the indicated concentrations of sphingosine. Cell viability was determined by the MTT assay, indicated as a percentage of absorbance observed in the absence of sphingosine. The indicated results are the means and SD of triplicate values obtained in one representative experiment of four.

Watabe-Uchida et al., 1998; Maeno et al., 1999). The restoration of cadherin-dependent cell adhesion was occasionally accompanied by the retardation of cell growth (Watabe et al., 1994; Bullions et al., 1997), a reduced ability to form colonies in vitro (Ewing et al., 1995; Bullions et al., 1997), the suppression of tumorigenesis in vivo (Ewing et al., 1995; Bullions et al., 1997), and the assembly of apical junctional complexes, including tight junctions (Watabe et al., 1994; Watabe-Uchida et al., 1998). The restoration of α -catenin expression in DLD-1/ $\Delta\alpha$ cells, an α -catenin-deficient colon carcinoma cell line, resulted in an epithelioid morphotype but did not induce growth suppression. After the induction of cell death by exogenous stimuli, such as sphingosine, the expression of α -catenin reduced cell death within a critical concentration range of the inducer. This reduction of cell death resulting from α -catenin expression was also observed in PC 3 cells, an additional α -catenin-deficient cell line derived from a human prostate cancer. This is the first report of a reduction of cell death induced by the expression of α -catenin.

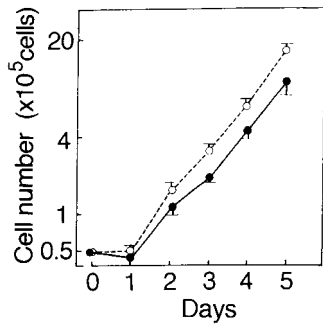


Figure 10. **Effect of α -catenin expression on cell growth.** nD cells (●) and α D cells (○) were seeded at an initial density of 5×10^4 cells per 35-mm dish. Cell numbers were then determined over a period of 5 d, after trypsin/EDTA treatment. The values are the means and SD of triplicate counts.

In the present study, we could not directly measure the rate of entry into apoptosis because of the difficulties to quantify nuclear fragmentation of DLD-1/ $\Delta\alpha$ transfectants. Cells undergoing cell death detached from dish and centrifugation to collect and to stain the cells destroyed large amount of floating cells. However, as described in Results several lines of evidence, (a) DNA fragmentation, (b) nuclear fragmentation, and (c) activation of caspase, indicate that apoptosis is taking place in our experiment and thus support the correlation between reduced viability and apoptosis.

Linkage to the cytoskeleton but not cadherin-mediated change in morphology itself is correlated with resistance to sphingosine-induced cell death

The transfection of cells with α -catenin deletion mutants demonstrated that α -catenin mutants capable of association with the actin cytoskeleton can induce compaction in three-dimensional suspension cultures; these mutants also exhibit an anti-cell death function. These results indicate that the linkage of the cadherin-catenin complex to the cytoskeleton is integral in the induction of resistance to cell death. These mutants induce increased cell-cell contact when the transfectants are cultured on solid substrate, the conditions used in cell death experiments. Disruption of cell adhesion by treatment with neutralizing anti-E-cadherin antibodies did not reduce the resistance to cell death, although the cadherin-mediated tight adhesion was lost from α D cells. Therefore, the increased cell-cell contact does not appear to be responsible for the increased viability. This phenotype is induced at the same time as the resistance to cell death but induced independently by α -catenin expression. Further studies are needed to clarify the molecular differences between cells lacking α -catenin and cell treatment with an anticadherin antibody.

Both the middle and COOH-terminal regions may link the cadherin-catenin complex and the actin cytoskeleton via different proteins. This redundancy may explain why the effect of α -catenin on cell death is not specific for either domain of the molecule. The middle region of the molecule, amino acids 203–381, overlaps with the vinculin-binding region reported to be contained within amino acids 326–509 (Watabe-Uchida et al., 1998, Imamura et al., 1999). In

addition, amino acids 325–394 have been identified as an α -actinin-binding site (Nieset et al., 1997). The vinculin- and/or α -actinin-mediated linkage could function in $\alpha\Delta$ C4D cells. The COOH-terminal 210 residues, amino acids 697–906, interact directly with actin filaments in vitro (Weiss et al., 1998). In addition, amino acids 697–906 of α -catenin bind to ZO-1 (Imamura et al., 1999). In $\alpha\Delta$ NM1D cells, linkages to the actin cytoskeleton could be established through direct binding or indirect binding via ZO-1. These two types of linkage may simultaneously influence death induction and induce close cell-cell contacts.

p27kip1 as a potential mediator of the resistance to cell death dependent on α -catenin

p27Kip1 may mediate the signal(s) linking the cadherin-catenin complex to the apoptotic cascade. This molecule, originally identified as a cdk inhibitor involved in G1 phase arrest signaled by TGF β and cell-cell contact (Polyak et al., 1994), may transduce the signal(s) from the cadherin-catenin complex, resulting in growth suppression (St. Croix et al., 1998). However, these results could not be applied directly to our study, since E-cadherin-dependent increases in p27kip1 were reported to be observed only in three-dimensional suspension cultures not in monolayer cultures. We examined p27kip1 levels to discover that protein levels were upregulated in low density cultures of α D cells compared with those observed in nD cells. The cause of the discrepancy between our results and the previous ones is not clear; differences in the cell line, cell density, or adhesive disruption method used may explain these differences. They used transfection of E-cadherin and its neutralizing antibodies, whereas we used a deficiency of α -catenin. p27kip1 protein levels were as high as the levels observed in α D cells in transfectants expressing α -catenin deletion mutants that confer resistance to cell death.

Transfection-mediated overexpression of p27kip1 decreases sensitivity to apoptosis induced by DNA-damaging drugs (Dimanche-Boitrel et al., 1998; Eymin et al., 1999a,b). In leukemic cells, p27kip1 overexpression inhibits cytochrome c release from mitochondria and procaspase 3 activation (Eymin et al., 1999a,b). Release of cytochrome c from mitochondria is controlled by proteins of the Bcl family; proapoptotic members of the family, Bax and Bak, accelerate the opening of mitochondrial permeability transition pore, whereas antiapoptotic members of the family, such as Bcl-xL, inhibit (Shimizu et al., 1999). Increases in permeability result in the relocation of cytochrome c from mitochondria to the cytosol, aiding the activation of caspases (Li et al., 1997). These results suggest that p27kip1 may prevent cell death upstream of mitochondrial events, possibly through Bcl family proteins. In this study, analysis of the antiapoptotic Bcl family and the death mediator caspases demonstrated that the target(s) facilitating the resistance of α D cells to death may function upstream of Bcl-xL and/or caspase 3 (or caspase 3-like proteases). This is consistent with the idea that p27kip1 may link the signal(s) mediated by the cadherin-catenin complex to death mediator molecules involved in sphingosine-induced cell death. Finally, increased expression of p27kip1 in α -catenin-deficient (DLD-1/ $\Delta\alpha$) cells decreases the induction of cell death by sphingosine.

The precise mechanism whereby p27kip1 affects the cell death cascade remains unknown; two possible mechanisms may mediate this effect dependent or not dependent on cell cycle progression. In the present study, α -catenin expression did not suppress cell growth; therefore, a cell cycle-dependent mechanism does not appear likely. In addition, caspase-mediated cleavage of p27kip1 into a 15-kD NH₂-terminal fragment is required for antiapoptotic activity (Eymin et al., 1999b). A point mutation of amino acid 108 (D to E), the putative cleavage site for the caspase, resulted in both resistance to cleavage and the loss of the antiapoptotic function, preventing both the release of cytochrome c from the mitochondria and the activation of caspase 3 (–like proteases). These findings suggest that p27kip1 possesses an unknown function, independent of the inhibition of cell cycle progression. The molecular mechanism(s) underlying the anti–cell death function remains to be investigated.

Recently, Vasioukhin et al. (2001) showed that α -catenin deletion induced increased cell proliferation and downregulation of p27kip1 but not elevated apoptotic rate. We do not know why downregulation of p27kip1 does not lead an elevated apoptosis in their system. One possible explanation could be the difference in the cell types used in the experiments, that is, normal keratinocytes in their experiments versus cancer cells in ours.

We demonstrated that α -catenin expression increases resistance to cell death. The deletion mutants of α -catenin, capable of increasing cell–cell adhesion, can also decrease the susceptibility to cell death after sphingosine treatment. However, the anti–cell death function does not require cell–cell contact itself as indicated by treatment with anti–E-cadherin antibodies. The α -catenin–induced increases in p27kip1, a cdk inhibitor, may mediate the anti–cell death function.

Materials and methods

Chemicals and antibodies

D-Sphingosine (sulfate salt), MTT, mouse IgG, and an antivinculin mAb were purchased from Sigma-Aldrich. An mAb against Bcl-2 was obtained from Santa Cruz Biotechnology, Inc. Polyclonal antibodies against Bcl-xL and mAbs against α -catenin, p27kip1, and p21cip1 were purchased from Transduction Laboratories. An mAb against HA (3F10) was obtained from Roche Molecular Biochemicals. SHE78-7, an mAb against human E-cadherin, was purchased from Takara Shuzo, Co. Both a colorimetric tetrapeptide substrate, acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA), and a specific inhibitor, acetyl-Asp-Glu-Val-Asp-chloromethylketone (Ac-DEVD-cmk), of caspase 3 (–like proteinase) were obtained from Calbiochem.

Cells and transfection

DLD-1/ $\Delta\alpha$ cells, a DLD-1 human colon cancer cell line variant, were provided by Dr. S.T. Suzuki (Aichi Human Service Center, Kasugai City, Aichi, Japan). Cells were grown and transfected as described previously (Ozawa, 1998). G418-resistant clones were examined by immunoblotting for the expression of either α -catenin or the various deletion mutants. Positive cells were subcloned; a representative for each construct from multiple transfectant clones was chosen to be used for further studies.

For multicellular suspension cultures, cells were plated in 35-mm dishes coated previously with polyhydroxyethylmethacrylate (Frisch and Francis, 1994).

Sphingosine treatment

Cells were seeded at 5×10^4 cells in 35-mm dishes containing 2 ml culture medium and incubated for 24 h. To induce apoptosis, culture medium containing either sphingosine or the drug vehicle (DMSO) alone was dispensed into the dishes. The culture dishes were then incubated for 3–24 h

before analysis. Cells were seeded at 2,500 cells per well in 96-well plates in 100 μ l of culture medium containing either SHE78-7 or control IgG, then treated as above for the experiments using an anti–E-cadherin–blocking antibody.

Cell viability studies

Cell viability after sphingosine treatment was assessed using the MTT assay described by Alley et al. (1988). Samples were incubated with MTT for 1 h; detached cells were then collected by centrifugation and combined with attached cells. Cell viability was expressed as a percentage of the control absorbance after subtracting the background absorbance of culture medium without cells.

DNA fragmentation assay

Nucleosomal DNA degradation was assayed as described by Sinha et al. (1995) with the following modifications. In brief, 2×10^5 cells were seeded in a 100-mm culture dish and treated with 17.5 μ M sphingosine for 18 h. After drug treatment, suspension cells were collected and centrifuged. The remaining cells were collected by trypsinization and combined with the suspension cells. After a wash with ice-cold PBS, cells were lysed for 10 min at 4°C in 100 μ l of lysis buffer comprised of 10 mM Tris (pH 7.4), 10 mM EDTA, and 0.5% Triton X-100. The lysate was centrifuged at 27,000 g for 20 min; the resulting supernatant was treated with RNase A (400 μ g/ml) for 30 min at 37°C, followed by treatment with proteinase K (400 μ g/ml) for 1 h at 37°C. DNA was precipitated with an equal volume of iso-propanol in the presence of 0.5 M NaCl overnight at –20°C. Total DNA from each sample was visualized on a 2% agarose gel.

DAPI staining

Cells were grown on coverslips and stained according to the method of the manufacturer (Roche Diagnostics GmbH). In brief, the sphingosine-treated cells were washed once with DAPI-methanol (1 μ g/ml) and then incubated in the same solution for 15 min at 37°C. After washing once with methanol, cells were examined under a fluorescence microscope with a 330–385-nm excitation filter.

Immunoblotting

Before protein analysis, cells were seeded at 2×10^5 per 100-mm dish in 8 ml culture medium. Culture medium containing sphingosine was dispensed after a 48-h incubation to induce apoptosis. Preliminary experiments demonstrated that cell viability after sphingosine treatment was similar to that of cells treated in 35-mm dishes as described above. After drug treatment, floating cells were centrifuged. After two washes with ice-cold PBS, we added SDS sample buffer (Laemmli, 1970) to both the cells collected by centrifugation and those attached to the dish. Cells were lysed, combined, and boiled for 5 min. After SDS-PAGE (12.5% polyacrylamide), proteins were transferred to nitrocellulose membranes. Proteins were detected as described previously (Ozawa, 1998) with the following antibodies: anti–Bcl-2 (0.2 μ g/ml), anti–Bcl-xL (0.5 μ g/ml), anti– α -catenin (1.25 μ g/ml), anti-HA (0.2 μ g/ml), anti-p27kip1 (0.5 μ g/ml), anti-p21cip1 (0.25 μ g/ml), and antivinculin (8.4 ng/ml). Antivinculin antibodies were used to monitor fluctuations in the total protein amount. Blots were quantified with a scanner (Scan Jet 4c/T; Hewlett Packard) and NIH Image 1.62 f software.

Measurement of caspase 3 (–like) activity

Cells were treated with sphingosine in 100-mm dishes as described above. After harvesting and washing, cells were resuspended in a hypotonic cell lysis buffer (20 mM Hepes, pH 7.5, 10 mM KCl, 2mM MgCl₂, 2 mM DTT, 2mM PMSF, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin) and lysed by four cycles of freeze-thaw. Cell lysates were centrifuged for 20 min at 16,000 g; the resulting supernatant was used as the source of enzyme. Caspase 3 (–like) activity was measured with a colorimetric tetrapeptide, Ac-DEVD-pNA, in the presence (negative control) or absence (assay) of the caspase 3 inhibitor Ac-DEVD-cmk as described by Datta et al. (1997). The activity was calculated to be the difference between the 405-nm absorbance of the assay mixture and the negative control.

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