BIOLOGY OF REPRODUCTION **64,** 1183–1190 (2001)

E-Cadherin-Mediated Cell Contact Prevents Apoptosis of Spontaneously Immortalized Granulosa Cells by Regulating Akt Kinase Activity[1]

J.J. Peluso,[2,3,4] A. Pappalardo,[3] and G. Fernandez[3]

Departments of Physiology[3] and Obstetrics and Gynecology,[4] University of Connecticut Health Center, Farmington, Connecticut 06030

ABSTRACT

The present studies were designed to determine the role that homophilic E-cadherin binding plays in preventing apoptosis of spontaneously immortalized granulosa cells (SIGCs). Although the levels of E-cadherin were similar to serum control levels, the amount of E-cadherin at the plasma membrane was dramatically reduced by 5 h after serum withdrawal. To determine whether disrupting homophilic E-cadherin binding leads to apoptosis, SIGCs were cultured in serum in the presence of either EGTA or an E-cadherin antibody. Treatment with either EGTA, which disrupts all calcium-dependent contacts, or E-cadherin antibody, induced apoptosis. Exposure to EGTA reduced MEK and Akt kinase activity, whereas E-cadherin antibody only attenuated Akt kinase activity. Because Akt kinase controls caspase-3 activity, an important activator of apoptosis, caspase-3 activity was monitored. Caspase-3 activity increased after serum depletion, or EGTA or E-cadherin antibody treatment. Time-series analysis of caspase-3 activity within single cells revealed that during apoptosis cell contact was disrupted then caspase-3 activity was detected. Finally, the caspase inhibitor, Z-VAD-FMK, blocked apoptosis. These data taken together are consistent with the concept that E-cadherin-mediated cell contact, either directly or indirectly, promotes Akt kinase activity, which in turn, inhibits caspase-3 activation and thereby maintains SIGC viability.

apoptosis, follicle, granulosa cells, growth factors, kinases, ovary, signal transduction

INTRODUCTION

It has been known for several decades that the gonadotropins, LH and FSH, regulate follicular development by determining which follicles develop and which become atretic [1–3]. Gonadotropins promote follicular development by regulating granulosa cell mitosis and apoptosis [4, 5]. In large part, gonadotropins mediate their action by stimulating the expression of various ovarian-derived growth factors. These ovarian-derived growth factors include such diverse factors as insulin-like growth factor-1, growth hormone, epidermal growth factor, hepatocyte growth factor, and basic fibroblast growth factor (bFGF), as well as estrogen and progesterone [4, 5]. These growth factors, acting in an autocrine manner, a paracrine manner, or both, activate various signal transduction pathways that control mitosis, cell survival, or both.

In addition to ovarian-derived growth factors, cellular interactions with the extracellular matrix and other cells influence granulosa cell function. For example, interaction with extracellular matrix proteins regulates granulosa cell morphology, steroidogenic potential, and gonadotropin responsiveness [6–8]. Similarly, cadherin-mediated cell contact enhances the ability of FSH to regulate granulosa cell function [9–11]. The effects of cell-cell and cell-matrix interactions are likely due in part to their ability to promote cell viability [6, 12, 13].

How interactions with the extracellular matrix or other cells activate signal transduction cascades that inhibit apoptosis has not been clearly defined. It is known that both the mitogen-activated protein kinase (MAPK) and the phoshatidylinositol 3-kinase (PI-3K)-Akt kinase signal transduction pathways regulate cell survival [14, 15]. Whether these pathways are stimulated as part of the anti-apoptotic action initiated by homophilic cadherin binding in granulosa cells has not been determined. To address this issue, we have utilized a spontaneously immortalized granulosa cell (SIGC) line. These cells lose contact with other cells and subsequently undergo apoptosis when deprived of serum or bFGF [16]. The present studies were designed to determine the relationship between the loss of cell-cell contact, the levels and cellular location of the adhesion proteins, E-cadherin and β-catenin, the integrity of the actin cytoskeleton, and apoptosis. Once these relationships were established, the effects of distributing E-cadherin-mediated cell contact on the activity of MEK kinases, the kinases that phosphorylate MAPK [14, 15] and Akt kinase, were assessed. Finally, caspase-3 activity was monitored in both populations of cells and in individual cells undergoing apoptosis.

MATERIALS AND METHODS

SIGC Culture

SIGCs were generously provided by Dr. Robert Burghardt of Texas A&M University (College Station, TX) and cultured as previously described [16]. The SIGCs were routinely maintained in Falcon T-flasks (Becton Dickinson Labware, Lincoln Park, NJ). For experiments involving Western blots or caspase-3 activity, the SIGCs were plated in 100-mm glass (Kimax) culture dishes at a density of 4×10^5 cells/ml in 5 ml of medium. In experiments in which apoptosis was assessed by YOPRO-1 staining, SIGCs were plated in 0.4 ml of medium at 1.25×10^5 cells/ml in 8-chamber glass lab-tek slides (Nunc Inc., Naperville, IL). For studies involving the localization of E-cadherin, β-catenin, actin, or caspase-3 activity, SIGCs were plated on round cover slips that were placed in 35-mm culture dishes. Regardless of the culture vessel, the cells were initially cultured in Dulbecco modified Eagle medium (DMEM)/F-12 supplemented with 5% fetal bovine serum for 24 h. The serum-supplemented medium was removed and the cells were cultured in serum-free DMEM/F-12 for 5 h unless stated otherwise. Depending on the experimental design the DMEM/F-12 was supplemented with EGTA (9 nM), im-

[1]Supported by National Institutes of Health grant HD 33467.
[2]Correspondence. FAX: 860 679 1269; e-mail: peluso@nso2.uchc.edu

Received: 25 August 2000.
First decision: 27 September 2000.
Accepted: 21 November 2000.

ISSN: 0006-3363. http://www.biolreprod.org

munoglobulin G (IgG; 2 μg/ml, Sigma Chemical Co., St. Louis, MO), or E-cadherin antibody (2 μg/ml; DECMA-1, Sigma), which is known to block homophilic E-cadherin binding [17]. The IgG and E-cadherin antibody were dialyzed against PBS for 24 h at 4°C as described in [17]. The caspase inhibitor, Z-VAD-FMK (Promega, Madison, WI), was added at a final concentration of 50 μM.

Identification of Apoptotic Cells

Apoptosis was assessed by in situ staining using the impermeant nuclear dye, YOPRO-1. To stain apoptotic cells, YOPRO-1 was added directly into each culture chamber at a final concentration of 10 μM. The cells were incubated for 10 min at 37°C and then observed at a magnification of 200× under fluorescent optics using the fluorescein-isothiocyanate conjugated (FITC) filter set. The number of fluorescent cells (i.e., apoptotic cells) in a field was counted. The total number of cells in that field was counted under phase optics. A total of 100–200 cells per well were counted. The percentage of apoptotic cells was then calculated [16].

Western Blot Analysis

Preparation of cell lysates. After treatment, late stage apoptotic cells (i.e., loosely attached cells) were separated from the attached cells as previously described [16]. Cell lysates were prepared from both the attached and loosely attached cells after the various treatments [16]. Protein determinations were made using the BCA method (Pierce, Rockford, IL). Typically, 10 μg of lysate was loaded onto each lane and the sample was electrophoresed on a 10% polyacrylamide gel at 100 V. Proteins were then transferred to nitrocellulose and incubated with 5% nonfat milk (Nestlé Food Company, Glendale, CA) in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 h with agitation at room temperature.

E-Cadherin and β-catenin detection. The nitrocellulose blot was then incubated for 2 h with agitation at room temperature with either a 1:2500 dilution of an E-cadherin monoclonal antibody (Transduction Laboratory, Lexington, KY) or a 1:500 dilution of a monoclonal β-catenin antibody (Transduction). The blots were washed four times with 1% nonfat milk in TBS-T and incubated with a 1:25 000 dilution of a peroxidase-labeled goat anti-mouse IgG (Kirkegaard and Perry, Gaithersburg, MD) for 1 h with agitation at room temperature. The specific protein was detected by chemiluminescence using the SuperSignal ULTRA detection system (Pierce). Specific staining was assessed by omitting the primary antibody from the Western blot protocol.

Membrane-associated E-cadherin. To determine the amount of E-cadherin that was associated with cell membrane, the plasma membrane proteins were biotinylated using the EZ-link sulfo-NHS-LC-LC-Biotin reagent and protocol provided by Pierce [18]. Once these proteins were biotinylated, cell lysates were prepared. A sample of this lysate was assessed for the total amount of E-cadherin by Western blot. To isolate the plasma membrane proteins, the remainder of the sample was immunoprecipitated using Ultralink Immobilized streptavidin using the protocol provided by Pierce. Twenty micrograms of the immunoprecipitate was then run on a 10% acrylamide gel and E-cadherin that was present at the plasma membrane was determined by Western blot as previously described. In addition, 5 μg of sample was electrophoresed and probed with a streptavidin-horseradish peroxidase conjugate to detect plasma membrane proteins.

Localization of Actin, E-Cadherin, and β-Catenin

The actin cytoskeleton was assessed by staining formalin-fixed cells with phalloidin-TRITC as described by Wieland [19]. E-Cadherin that was localized to the plasma membrane was detected by staining living cells as outlined in [20]. Briefly, cells were rinsed with PBS and then incubated in the presence or absence of E-cadherin antibody (DECMA-1, 1:50 in 8% BSA/PBS; Sigma) for 15 min at room temperature. After this incubation, the cells were washed in PBS and incubated with FITC-IgG (1:100 in 8% BSA/PBS; Sigma) for 15 min in the dark at room temperature. The cells were then washed with PBS and observed under phase and fluorescent optics.

To localize β-catenin, the formalin-fixed cells were permeabilized with 1% Triton-X. The cells were then incubated with a 1:50 dilution of the anti-β-catenin antibody (Transduction) for 2 h at room temperature, washed four times in PBS, and incubated with a 1:100 dilution of FITC-labeled goat anti-mouse IgG for 1 h at room temperature. The β-catenin-stained coverglasses were washed four times in PBS, stained with propidium iodide, mounted onto slides, and observed under a confocal microscope. Specific staining was assessed by omitting the primary antibody from the protocol.

Assessment of MEK 1/2, and Akt Activity

The level and activity of MEK 1/2 was assessed using reagents and protocols provided by New England Biolabs (Beverly, MA). Briefly, the total amount of MEK was detected using a monoclonal antibody, whereas the amount of activated MEK was determined using a monoclonal antibody that selectively recognizes active MEK (i.e., phosphorylated MEK at Ser 217/221). The antibodies used in these studies detected both MEK 1 and 2.

Similarly, Akt kinase levels and activity were detected using the reagents and protocols provided by New England Biolabs. Basically, a Western blot using a monoclonal antibody was used to estimate the total amount of Akt. This antibody, which recognizes all three forms of AKT kinase, was also used to immunoprecipitate Akt kinase. The resulting immunoprecipitate was then incubated with GSK-3 fusion protein in the presence of ATP and kinase buffer. Because Akt kinase phosphorylates GSK-3, the amount of Akt kinase activity was estimated by determining the amount of phosphorylated GSK-3 by Western blot using a phospho-specific antibody.

The levels and activities of both MEK 1/2 and Akt were quantitated by determining the density of the appropriately sized band using IP Lab Gel software (Scanalytics, Fairfax, VA). The relative amount or activity of each kinase was expressed as a percentage of the appropriate control value for each experiment.

Caspase-3 Activity

Quantitative measurements. Caspase-3 activity was determined using a colorimetric assay kit provided by Promega. In this assay, caspase-3 activity is determined by the amount of chromophore (pNA) that is released from the caspase-3 substrate, Ac-DEVD-pNA. The relative level of

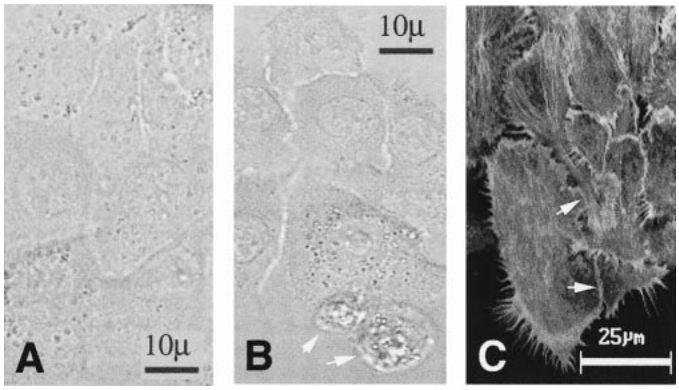

FIG. 1. The effect of serum depletion of the morphology and actin cytoskeleton of SIGCs. Phase micrographs of SIGCs cultured in serum (**A**) or 5 h after serum withdrawal (**B**) are shown. Compared with SIGCs grown in serum, the SIGCs cultured for 5 h in serum-free medium are separated from each other. The white arrows in **B** identify ''loosely attached'' SIGCs that are apoptotic and can be removed by aspiration. In **C**, the actin cytoskeleton of SIGCs in the early stages of apoptosis is shown. The actin cytoskeleton consists of stress fibers and cortical actin with the actin cytoskeleton firmly anchored to the extracellular matrix.

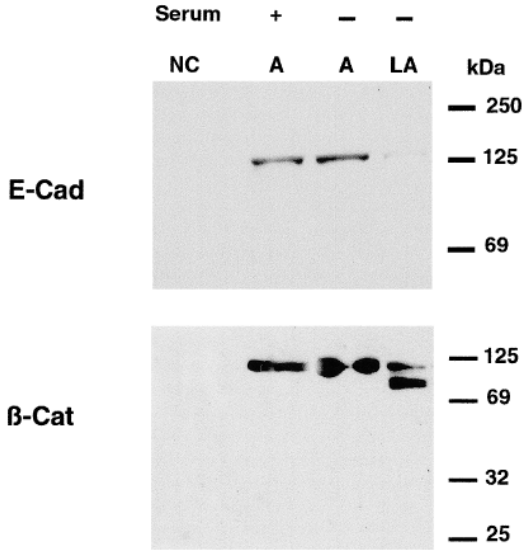

FIG. 2. The effect of serum depletion on E-cadherin (E-cad) and β-catenin (β-cat) levels. SIGCs cultured in the absence of serum were separated into populations of attached (A) and loosely attached (LA) cells. Negative control (NC) is shown in the first lane of this figure.

caspase-3 activity was expressed as a fold increase compared with serum or IgG control values.

Single-cell assessment of caspase-3 activity. In this assay, SIGCs were incubated in either serum-containing or serum-free Krebs-Hepes buffer supplemented with a caspase-3 substrate, PhiPhiLux G_1D_2 (9 μM; Calbiochem, San Diego, CA). This substrate contains the sequence GDEVDGI, and does not fluoresce. However, when cleaved by caspase-3, it has a green fluorescence. To observe the relationship between cell contact and caspase-3 activity, SIGCs were cultured for 5 h at 37°C and observed under both phase and fluorescent optics at 0.5-h intervals.

Statistical Analysis

All experiments were repeated at least two to three times with each experiment yielding essentially identical results. Each experiment in which apoptosis was assessed by YOPRO-1 staining was done in quadruplicate. These data were pooled and analyzed by a one-way ANOVA followed by a Student-Newman-Keuls test, when appropriate. *P* values of less than 0.05 were considered to be significant.

RESULTS

When cultured in serum-supplemented medium, SIGCs formed tight contacts along their entire cell boarder (Fig. 1). After serum withdrawal some cells were not attached to the extracellular matrix (i.e., the culture dish) and only ''loosely attached'' to other cells (Fig. 1). These cells had disorganized nuclei and were round, suggesting that their cytoskeleton had collapsed. The collapse of the actin cytoskeleton was confirmed by phalloidin staining (data not shown). These loosely attached cells were removed by aspiration from the culture dish. The remaining cells maintained their shape and interaction with the extracellular matrix but showed a reduction in cell-to-cell contact as observed under phase optics. These cells possessed an intact actin cytoskeleton with numerous actin stress fibers. The actin cytoskeleton was firmly anchored to the extracellular matrix (Fig. 1). Previous studies have shown that the attached cells were undergoing apoptosis because they bind annexin V and ultimately undergo apoptosis by 24 h of serum deprivation [16].

The adhesion proteins, E-cadherin and β-catenin, were readily detected by Western blot in SIGCs grown in serum-supplemented medium (Fig. 2). After serum withdrawal, levels of E-cadherin were dramatically reduced in the loosely attached cells. Intact as well as degradation fragments of β-catenin were detected in the loosely attached cells. For those cells that remained attached after serum withdrawal, both E-cadherin and β-catenin levels were maintained at serum control levels (Fig. 2).

As shown in Figure 3A, several biotinylated plasma membrane proteins were detected in SIGCs cultured in serum. Most of these proteins remained at the plasma membrane after serum withdrawal. It is interesting that E-cad-

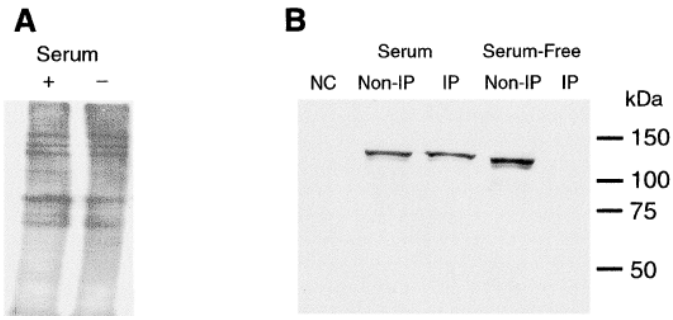

FIG. 3. The effect of serum withdrawal on the levels of E-cadherin at the plasma membrane. In this study, cells were cultured in the presence or absence of serum. After culture, the loosely attached cells were discarded. Using the attached cells, the surface proteins were biotinylated as described in *Materials and Methods*. Lysates were then probed with streptavidin conjugated with horseradish peroxidase to detect the biotinylated proteins (**A**). Lysates were used in either an E-cadherin Western blot (i.e., nonimmunoprecipitated (Non-IP) or immunoprecipitated (IP) with streptavidin-coated beads to isolate all the proteins present at the surface membrane (**B**). As shown in **A**, many proteins are present at the surface membrane of SIGCs grown in serum. Most of these proteins were still present after serum withdrawal. Panel **B** demonstrates that although still present in SIGCs after serum withdrawal (compare Non-IP lanes), E-cadherin is not localized to the plasma membrane (compare IP lanes).

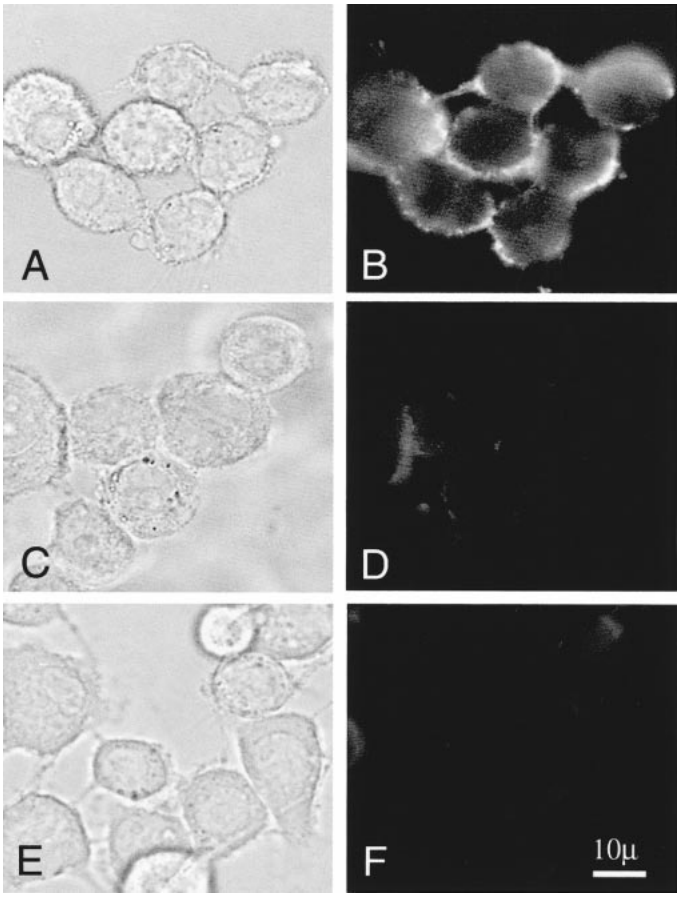

FIG. 4. Immunocytochemical localization of E-cadherin at the surface membrane of living SIGCs cultured in the presence (**A**, **B**, **E**, **F**) or absence of serum (**C**, **D**). Phase images are shown in **A**, **C**, and **E**, whereas specific fluorescence associated with E-cadherin is shown in **B** and **D**. Nonspecific fluorescence is shown in **F**.

herin was not detected at the plasma membrane of cells that remained attached after serum withdrawal, although the total E-cadherin level was equal to that of the serum controls (Fig. 3). This observation was confirmed by immunocytochemically staining living SIGCs (Fig. 4). Confocal analysis also revealed that most of the β-catenin was localized to the plasma membrane in the presence of serum but was detected throughout the cytoplasm and nucleus after serum withdrawal (Fig. 5).

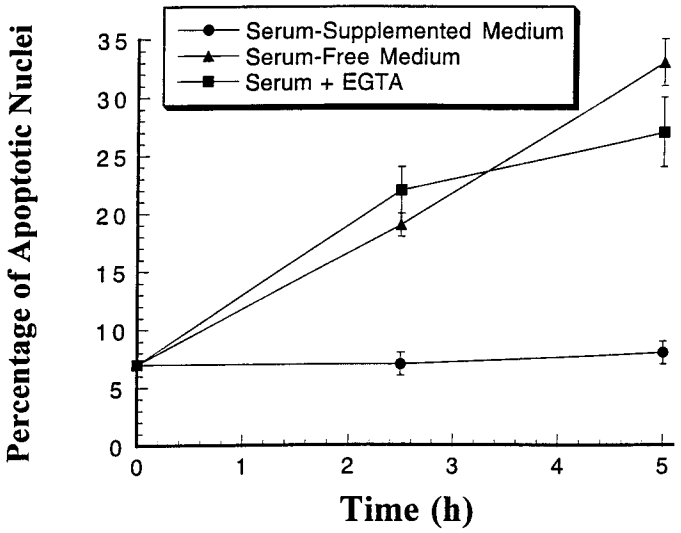

FIG. 6. The effect of serum, serum-free medium, or serum-containing medium supplemented with EGTA on SIGC apoptosis. In this study, SIGCs were cultured for 24 h in serum-supplemented medium then the cells were either 1) maintained in serum-containing medium, 2) placed in serum-free medium, or 3) cultured in serum-containing medium to which EGTA was added. Two-and-a-half and 5 h later, YOPRO-1 was added to the cultures and the percentage of apoptotic cells determined. The values represent the mean ± SEM.

To determine whether cell-cell interactions are involved in regulating SIGC survival, SIGCs were cultured with serum in the presence of EGTA. Under these conditions, all calcium-dependent cell-cell and cell-extracellular matrix contacts were disrupted. This study demonstrated that even in the presence of serum, EGTA induced SIGC apoptosis at a rate similar to that of the serum-free treatment (Fig. 6). In addition, cells that were plated for 5 h with serum in the presence of an E-cadherin antibody showed a slight reduction in cell-cell contact compared with the IgG-treated cells. However, the E-cadherin antibody-treated cells underwent

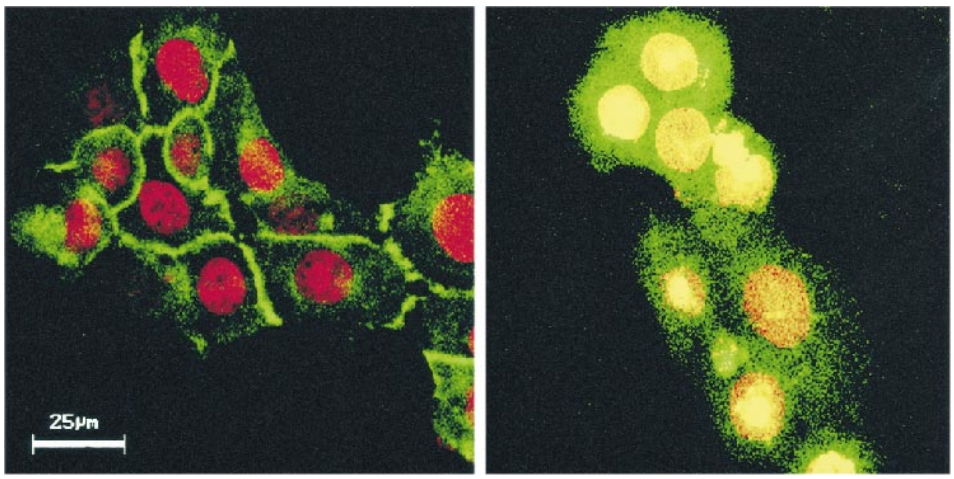

FIG. 5. Localization of β-catenin in SIGCs cultured in the presence (left panel) or absence (right panel) of serum. SIGCs were examined using a confocal microscope. Specific β-catenin staining is shown in green, whereas the DNA is stained red. The yellow staining represents areas where β-catenin colocalized within DNA. Under the optical setting used in this study, fluorescence was not detected in cells stained without the addition of the β-catenin antibody (i.e., the negative controls).

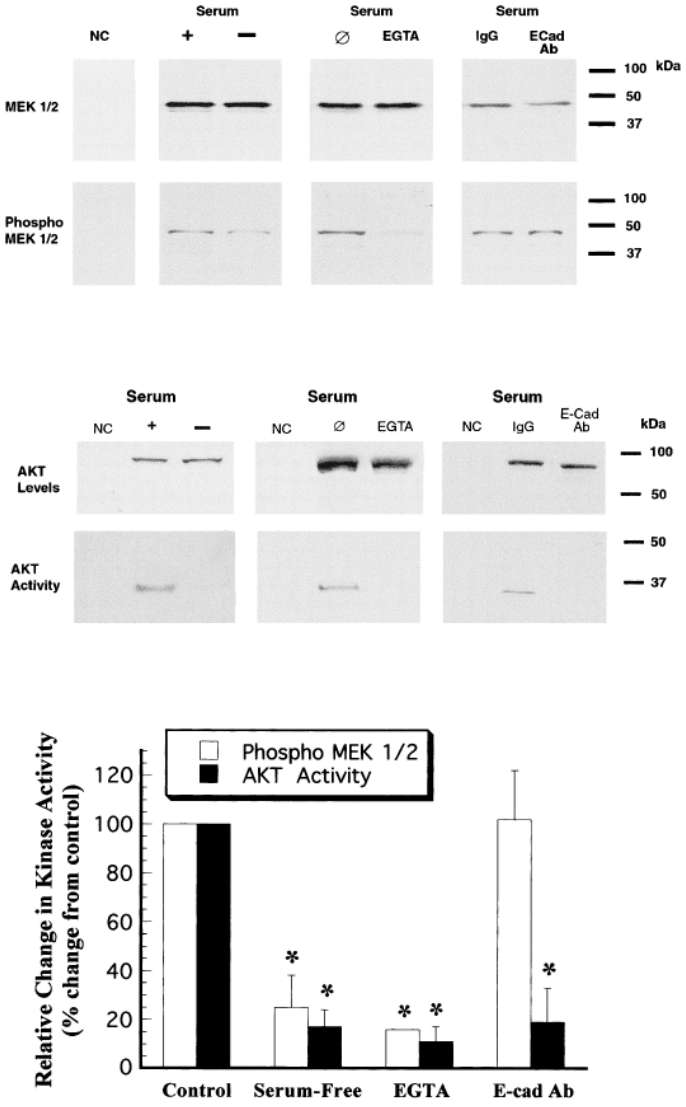

FIG. 7. The effect of disrupting cell contact on the levels and activity of MEK1/2 (upper panels) and Akt (middle panels) kinases. In these studies, cell contact was disrupted in three ways. First, SIGCs were cultured for 5 h in the presence (+) or absence of serum (−; second panel). For the second treatment, cells were plated in medium supplemented with serum (Ø) or serum and EGTA. The cells were harvested after 5 h. The third approach involved plating cells in serum-supplemented medium with either IgG or an antibody to E-cadherin (E-Cad Ab). Cells were also cultured for 5 h. Negative controls (NC) are shown. Relative changes in the activity of MEK and Akt are shown in the graph in the lower panel. *Values that are significantly less than control ($P < 0.05$).

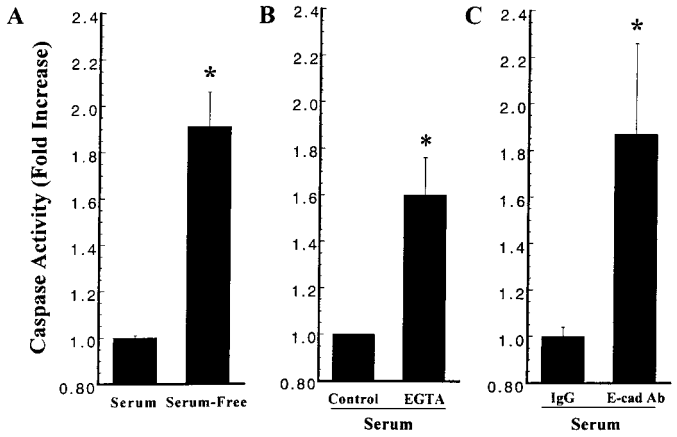

FIG. 8. The effects of serum withdrawal (**A**), EGTA (**B**), and E-cadherin antibody (**C**) on caspase-3 activity. SIGCs were cultured as in Figure 7. *Values that are significantly less than control ($P < 0.05$).

apoptosis at a rate nearly twice that of freshly harvested cells (15% ± 2% apoptotic cells) or cells plated with IgG (20% ± 2% for IgG vs. 35% ± 2% for E-cadherin antibody, $P < 0.05$; n = 12).

These findings are consistent with the concept that homophilic E-cadherin binding plays an essential role in activating the signal transduction pathways that ultimately control cell viability. One pathway that is often associated with cell survival is the MAPK pathway. MAPK is activated by MEK [14, 15]. As seen in Figure 7, MEK was present and activated (i.e., phosphorylated) in the presence of serum. Serum depletion or treatment with EGTA in the presence of serum significantly reduced MEK activity without altering MEK levels. In contrast, treatment with the E-cadherin antibody in the presence of serum did not affect either MEK levels or activity (Fig. 7).

Akt kinase or protein kinase B is another kinase that is also associated with cell survival [21]. Western blot analysis revealed that Akt kinase was expressed by SIGCs. Serum depletion and treatment with serum and either EGTA or E-cadherin antibody significantly reduced Akt activity without altering Akt levels (Fig. 7).

Because the activation of caspase-3 is inhibited by Akt kinase [21], studies were conducted to determine the effect of serum withdrawal or treatment with serum and either EGTA or E-cadherin antibody on caspase-3 activity. These studies revealed that caspase-3 activity was inversely related to Akt activity, increasing nearly twofold in response to serum depletion, EGTA, and E-cadherin antibody treatment (Fig. 8). To more clearly establish the relationship between cell-cell contact and caspase-3 activity, cells were cultured in serum-free medium supplemented with the caspase-3 substrate, PhiPhiLux G_1D_2. The cells were then sequentially observed. Within 1 h, cell-cell contact was disrupted, although the cells remained attached to the culture dish. After cell-cell contact was disrupted, caspase-3 activity was detected as judged by an increase in cell-specific fluorescence. This fluorescence was observed in both the cytoplasm and the nucleus (Fig. 9). The decrease in cell-cell contact and the increase in caspase-3 activity was not observed in cells maintained in serum-supplemented Krebs-Hepes buffer (data not shown). Finally, to determine whether caspase-3 activity ultimately leads to the apoptotic death of SIGCs, cells were cultured in serum-free medium in the presence or absence of the caspase inhibitor, Z-VAD-FMK. Under these conditions, the caspase inhibitor attenuated the rate of apoptosis induced by serum withdrawal or EGTA (Fig. 10, A and B).

DISCUSSION

In the later stages of apoptosis, SIGCs possess fragmented DNA and nuclei [16]. These late-stage apoptotic cells also lose contact with other cells as well as the extracellular matrix. In contrast, SIGCs in the early stages of apoptosis have intact DNA and a normal morphology [16]. These early stage SIGCs also possess an intact actin cytoskeleton that is firmly anchored to extracellular matrix. Although contact with the extracellular matrix is maintained, cell-cell contacts, as judged by phase microscopy, are re-

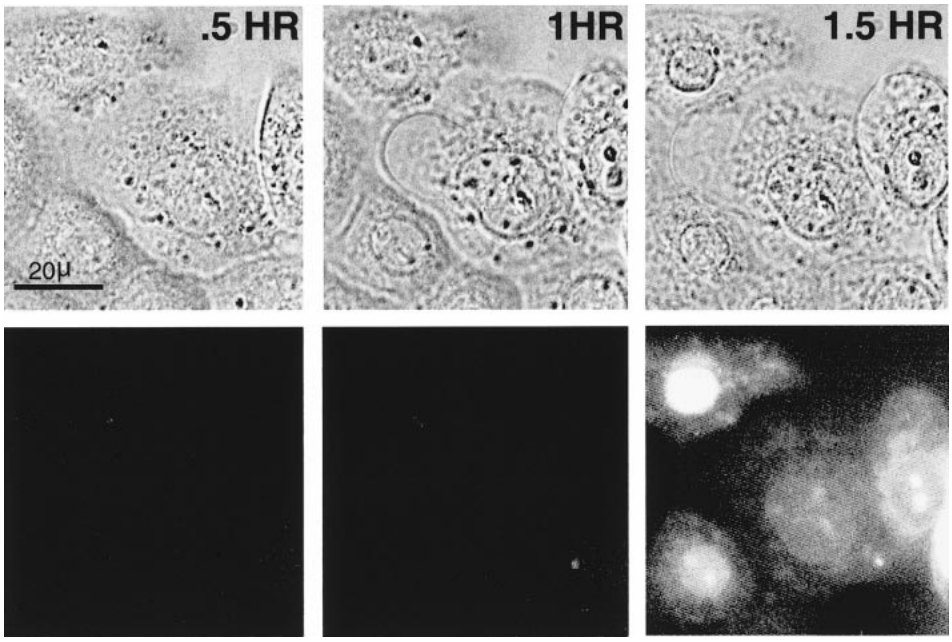

FIG. 9. The effect of serum withdrawal on caspase-3 activity within individual SIGCs. In this study, serum was removed and SIGCs cultured at 37°C in serum-free Krebs-Hepes buffer supplemented with the caspase-3 substrate, PhiPhiLux G_1D_2. Sequential phase and fluorescent images were collected at 0.5-h intervals over a 5-h culture period. In these cells, cell contact was disrupted by 1 h of culture, whereas caspase-3 activity was not detected until 1.5 h. Note the caspase-3 activity increased in both the cytoplasm and nucleus. SIGCs, maintained in Krebs-Hepes buffer supplemented with both serum and PhiPhiLux G_1D_2, served as controls. The controls did not show an increase in fluorescence during the 5-h culture period.

duced in SIGCs that are in the early stages of apoptosis. This suggests that a reduction in cell contact is an early event that could initiate the final events of the apoptotic cascade.

The present study also demonstrates that SIGCs express E-cadherin, which accounts in part for the calcium-dependent adhesion-type junctions that connect adjacent SIGCs [22]. It is likely that E-cadherin-mediated cell contact plays an important role in the mechanism through which cell contact regulates SIGC viability. This statement is based on the following observations: First, a reduction in cell contact precedes an increase in caspase-3 activity and apoptosis as judged by nuclear changes. Second, disrupting calcium-dependent cell contacts with either EGTA or an E-cadherin antibody results in SIGC apoptosis even in the presence of serum. It is important to appreciate that the overall rate of apoptosis in the IgG and E-cadherin antibody treatment groups was higher than the other studies. This was likely due to the fact that 15% of the cells were apoptotic at the time of plating and apoptosis was assessed in these studies only 5 h after plating and not after 24 h of culture with serum-supplemented medium.

The reduction in contact that is observed in early stage SIGCs is not due to E-cadherin degradation. Rather, the immunocytochemical and Western blot studies reveal that E-cadherin is not present at the plasma membrane of early stage SIGCs. There are several possible mechanisms that could account for E-cadherin not being at the plasma membrane of early stage SIGCs. First, because E-cadherin is normally associated with the actin cytoskeleton, subtle changes in the actin cytoskeleton could occur during the early stages of apoptosis that would disrupt this association. This is unlikely because early stage SIGCs possess an intact actin cytoskeleton and E-cadherin dissociates from actin only after both cortical actin and actin stress fibers have deteriorated [23]. The second possibility is that the extracellular domain of E-cadherin is cleaved. This cleavage is usually due to metalloproteinases [24]. This is also unlikely because a cytoplasmic E-cadherin fragments were not detected after serum withdrawal and the metalloproteinase in-

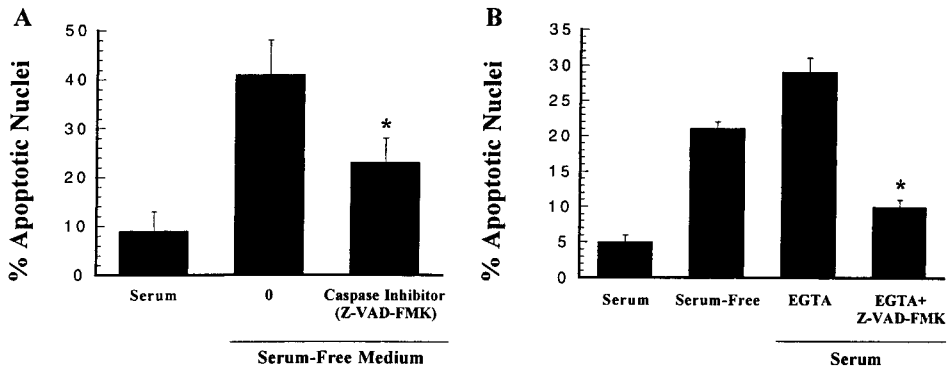

FIG. 10. The effect of the caspase inhibitor, Z-VAD-FMK, on the rate of apoptosis induced by serum withdrawal (**A**) or EGTA treatment (**B**). *Value significantly different from serum-free and/or EGTA treatment groups ($P < 0.05$).

hibitor, 1-10-phenanthroline, did not prevent SIGC apoptosis (unpublished observation). The third and most likely possibility is that in the early stages of SIGC apoptosis, E-cadherin is endocytosed. In healthy MDCK cells, E-cadherin is continuously internalized and recycled back to the plasma membrane [18]. It is possible that in the absence of serum-derived growth factors, E-cadherin is endocytosed but not returned to the plasma membrane. The stimuli for this endocytosis could be an increase in intracellular calcium ($[Ca^{2+}]_i$). In SIGCs, $[Ca^{2+}]_i$ increases within the minutes of serum withdrawal and is an essential event in the apoptotic cascade [16]. Moreover, elevated $[Ca^{2+}]_i$ promotes the formation of endocytotic vesicles [25] with internalized E-cadherin being detected in this type of endocytotic vesicles [23]. Finally, agents that increase $[Ca^{2+}]_i$ promote the redistribution of E-cadherin from the plasma membrane to the cytoplasm [24, 26].

The cellular location of the E-cadherin-associated protein, β-catenin, also changes in response to serum depletion. There are two interesting aspects regarding β-catenin localization. First, after serum withdrawal, β-catenin is detected within the cytoplasm and nucleus. Disrupting N-cadherin-mediated cell contact also results in an accumulation of β-catenin within the nuclei of rat ovarian surface epithelial cells [27]. In rat ovarian surface epithelial cells, β-catenin accumulates in the nucleus and subsequently stimulates the expression of genes that are required for these cells to undergo apoptosis [27]. It is not known whether β-catenin has the same effect in SIGCs.

The second interesting aspect is that cleavage products of β-catenin were detected in apoptotic SIGCs. Caspase-3 cleaves β-catenin into fragments of a similar size to that observed in apoptotic SIGCs [28–30]. E-Cadherin is also a substrate for caspase-3 [31]. Caspase-3 activity increases in both the cytoplasm and nuclei of SIGCs once cell-cell contact is disrupted. It is presumed that the increase in cytoplasmic caspase-3 activity accounts for the degradation of both β-catenin and E-cadherin. The elevated caspase-3 activity within the nucleus is likely to cleave nuclear proteins associated with the integrity of the DNA (PAK2, DNA-PK, and PARP) and the nuclear envelope (NuMa and Lamin) [32]. This proteolytic action of caspase-3 would ultimately result in DNA fragmentation, chromatin condensation, and the formation of apoptotic bodies.

In primary granulosa cells, caspase-3 promotes apoptosis [33–36]. Similarly, caspase-3 is also an important component in the apoptotic pathway of SIGCs because its activity increases during apoptosis and the caspase inhibitor, Z-VAD-FMK, blocks SIGC apoptosis. Furthermore, disruption of E-cadherin-mediated cell contact results in an increase in caspase-3 activity. This suggests that homophilic E-cadherin binding regulates a signal transduction pathway that inhibits the activation of caspase-3 and thereby promotes cell survival. At least two different signal transduction pathways, the Ras-MEK-MAP kinase pathway and the PI-3K-Akt kinase pathway, are associated with cell survival [14, 15] and therefore could be regulated by homophilic E-cadherin binding. MEK and Akt kinase were studied as representative members of these pathways. Both kinases are active in SIGCs cultured in serum and reduced in early stage SIGCs. Furthermore, disrupting all calcium-dependent interaction decreases the activity of both MEK and Akt kinase. However, the disruption of E-cadherin homophilic binding selectively reduces Akt kinase activity without influencing the MEK activity. As such, E-cadherin-mediated cell contact is likely to promote cell viability by promoting Akt kinase activity. It is interesting that the PI-3K/Akt kinase pathway has been shown to mediate the ability of IGF-1 to prevent porcine granulosa cell apoptosis [37]. Moreover, FSH has been shown to activate Akt kinase through a cAMP-dependent mechanism [38]. These findings, along the present findings, emphasize the importance of the PI-3K/Akt kinase pathway in preventing granulosa cell apoptosis.

Akt kinase controls several different pathways that promote cell survival [21]. One of its actions is to phosphorylate the proapoptotic Bcl-2 family member, Bad. In the phosphorylated state, Bad can not bind to the antiapoptotic agents, Bcl-2 or Bcl-xl. This allows Bcl-2, Bcl-xl, or both to bind Bax. This is important in that if Bax is not bound, it acts to induce the release of cytochrome c from the mitochondria, which in turn, leads to the activation of caspase-3 [39]. Therefore, by maintaining Akt kinase activity, homophilic E-cadherin binding would inhibit both caspase-3 activity and, subsequently, SIGC apoptosis.

How then could homophilic E-cadherin binding activate Akt kinase activity? In MDCK cells, homophilic E-cadherin binding recruits PI-3 kinase into an E-cadherin-containing protein complex where PI-3 kinase is activated through a growth factor-independent mechanism [40]. PI-3 kinase then leads to the activation of Akt kinase and thereby promotes cell survival [40]. A similar relationship between VE-cadherin, PI-3 kinase, Akt kinase, and apoptosis has also been described for endothelial cells [41]. In the absence of membrane-associated E-cadherin or as result of disrupting homophilic E-cadherin binding, PI-3 kinase may not be retained at the plasma membrane, thereby preventing its activation. This would result in reduced Akt kinase activity and subsequent apoptosis. Studies are presently being conducted to test this concept.

In summary, the present data are consistent with the hypothesis that homophilic E-cadherin binding functions to activate Akt kinase, which ultimately inhibits caspase-3 activity. By preventing the increase in caspase-3 activity, SIGC viability is maintained. Because E-cadherin is expressed by rat granulosa cells of primordial and primary follicles [42], E-cadherin-mediated cell contact is likely to be involved in preserving the viability of the granulosa cells of these small follicles. As follicular development continues, E-cadherin expression is suppressed and the expression of N-cadherin is increased [42]. N-Cadherin-mediated cell contact has been shown to promote the viability of rat [13, 43, 44] and human [12, 45] granulosa cells. Whether homophilic N-cadherin binding maintains granulosa cell viability by regulating Akt kinase remains to be determined.

ACKNOWLEDGMENTS

We thank Dr. Robert Burghardt of Texas A&M University for providing the SIGC cells and Dr. Bruce White for his helpful discussions regarding these studies.

REFERENCES

1. Hsueh A, Billig H, Tsafriri A. Ovarian follicle atresia: a hormonally controlled apoptotic process. Endocr Rev 1994; 15:707–724.
2. Hirshfield A. Development of follicles in the mammalian ovary. Int Rev Cytol 1991; 124:43–101.
3. Hsu SY, Hsueh AJW. Intracellular mechanisms of ovarian cell apoptosis. Mol Cell Endocrinol 1998; 145:21–25.
4. Chun SY, Eisenhauer KM, Minami S, Billig H, Perlas E, Hsueh AJ. Hormonal regulation of apoptosis in early antral follicles: follicle-stimulating hormone as a major survival factor. Endocrinology 1996; 137:1447–1456.

5. Billig H, Chun SY, Eisenhauer K, Hsueh AJ. Gonadal cell apoptosis: hormone-regulated cell demise. Hum Reprod Update 1996; 2:103–117.
6. Amsterdam A, Gold RS, Hosokawa K, Yoshida Y, Sasson R, Jung Y, Kotsuji F. Crosstalk among multiple signaling pathways controlling ovarian cell death. Trends Endocrinol Metab 1999; 10:255–262.
7. Asem EK, Feng SL, Stingley-Salazar SR, Turek JJ, Peter AT, Robinson JP. Basal lamina of avian ovarian follicle: influence on morphology of granulosa cells in-vitro. Comp Biochem Physiol [C] 2000; 125:189–201.
8. Ben-Rafael Z, Benadiva CA, Mastroianni L Jr, Garcia CJ, Minda JM, Iozzo RV, Flickinger GL. Collagen matrix influences the morphologic features and steroid secretion of human granulosa cells. Am J Obstet Gynecol 1988; 159:1570–1574.
9. Farookhi R, Desjardins J. Luteinizing hormone receptor induction in dispersed granulosa cells requires estrogen. Mol Cell Endocrinol 1986; 47:13–24.
10. Farookhi R, Geng CS, MacCalman CD, Blaschuk OW. Hormonal regulation of N-cadherin mRNA levels in rat granulosa cells. Ann N Y Acad Sci 1997; 816:165–172.
11. Harandian F, Farookhi R. Contact-dependent cell interactions determine hormone responsiveness and desensitization in rat granulosa cells. Endocrinology 1998; 139:1700–1707.
12. Makrigiannakis A, Coukos G, Blaschuk O, Coutifaris C. Follicular atresia and luteolysis—evidence of a role for N-cadherin. Ann N Y Acad Sci 2000; 900:46–55.
13. Peluso JJ. Putative mechanism through which N-cadherin-mediated cell contact maintains calcium homeostasis and thereby prevents ovarian cells from undergoing apoptosis. Biochem Pharmacol 1997; 54: 847–853.
14. Anderson P. Kinase cascades regulating entry into apoptosis. Microbiol Rev 1997; 61:33–46.
15. Lavin MF, Watters D, Song Q. Role of protein kinase activity in apoptosis. Experientia 1996; 52:979–994.
16. Lynch K, Ferandez G, Pappalardo A, Peluso JJ. Basic fibroblast growth factor inhibits apoptosis of spontaneously immortalized granulosa cells by regulating intracellular free calcium levels through a protein kinase c delta-dependent pathway. Endocrinology 2001; (in press).
17. Ozawa M, Kemler R. Altered cell adhesion activity by pervanadate due to the dissociation of alpha-catenin from the E-cadherin-catenin complex. J Biol Chem 1998; 273:6166–6170.
18. Le TL, Yap AS, Stow JL. Recycling of E-cadherin: a potential mechanism for regulating cadherin dynamics. J Cell Biol 1999; 146:219–232.
19. Wieland T. Peptides of poisonous amanita mushrooms. New York: Springer-Verlag; 1986.
20. Karecla PI, Green SJ, Bowden SJ, Coadwell J, Kilshaw PJ. Identification of a binding site for integrin alphaEbeta7 in the N-terminal domain of E-cadherin. J Biol Chem 1996; 271:30909–30915.
21. Franke TF, Kaplan DR, Cantley LC. PI3K: downstream AKTion blocks apoptosis. Cell 1997; 88:435–437.
22. Potter E, Bergwitz C, Brabant G. The cadherin-catenin system: implications for growth and differentiation of endocrine tissues. Endocr Rev 1999; 20:207–239.
23. Quinlan MP, Hyatt JL. Establishment of the circumferential actin filament network is a prerequisite for localization of the cadherin-catenin complex in epithelial cells. Cell Growth Differ 1999; 10:839–854.
24. Ito K, Okamoto I, Araki N, Kawano Y, Nakao M, Fujiyama S, Tomita K, Mimori T, Saya H. Calcium influx triggers the sequential proteolysis of extracellular and cytoplasmic domains of E-cadherin, leading to loss of beta-catenin from cell-cell contacts. Oncogene 1999; 18: 7080–7090.
25. Eddleman CS, Ballinger ML, Smyers ME, Fishman HM, Bittner GD. Endocytotic formation of vesicles and other membranous structures induced by Ca2+ and axolemmal injury. J Neurosci 1998; 18:4029–4041.
26. Pey R, Vial C, Schatten G, Hafner M. Increase of intracellular Ca^{2+} and relocation of E-cadherin during experimental decompaction of mouse embryos. Proc Natl Acad Sci U S A 1998; 95:12977–12982.
27. Peluso JJ, Pappalardo A, Hess SA. Effect of disrupting cell contact on the nuclear accumulation of β-catenin and subsequent apoptosis of rat ovarian surface epithelial cells in vitro. Endocrine 2000; 12:295–302.
28. Brancolini C, Schneider C. Cut and die: proteolytic cascades regulating apoptosis. Adv Clin Path 1997; 1:177–189.
29. Brancolini C, Lazarevic D, Rodriguez J, Schneider C. Dismantling cell-cell contacts during apoptosis is coupled to a caspase-dependent proteolytic cleavage of beta-catenin. J Cell Biol 1997; 139:759–771.
30. Steinhusen U, Badock V, Bauer A, Behrens J, Wittman-Liebold B, Dorken B, Bommert K. Apoptosis-induced cleavage of beta-catenin by caspase-3 results in proteolytic fragments with reduced transactivation potential. J Biol Chem 2000; 275:16345–16353.
31. Schmeiser K, Grand RJ. The fate of E- and P-cadherin during the early stages of apoptosis. Cell Death Differ 1999; 6:377–386.
32. Robertson JD, Orrenius S, Zhivotovsky B. Nuclear events in apoptosis. J Struct Biol 2000; 129:346–358.
33. Izawa M, Nguyen PH, Kim HH, Yeh J. Expression of the apoptosis-related genes, caspase-1, caspase-3, DNA fragmentation factor, and apoptotic protease activating factor-1, in human granulosa cells. Fertil Steril 1998; 70:549–552.
34. Johnson AL, Bridgham JT. Caspase-3 and -6 expression and enzyme activity in hen granulosa cells. Biol Reprod 2000; 62:589–598.
35. Robles R, Tao XJ, Trbovich AM, Maravel DV, Nahum R, Perez GI, Tilly KI, Tilly JL. Localization, regulation and possible consequences of apoptotic protease-activating factor-1 (Apaf-1) expression in granulosa cells of the mouse ovary. Endocrinology 1999; 140:2641–2644.
36. Boone DL, Tsang BK. Caspase-3 in the rat ovary: localization and possible role in follicular atresia and luteal regression. Biol Reprod 1998; 58:1533–1539.
37. Westfall SD, Hendry IR, Obholz KL, Rueda BR, Davis JS. Putative role of the phosphatidylinositol 3-kinase (PI3-kinase)-Akt signaling pathway in the survival of granulosa cells. Endocrine 2000; 12:315–321.
38. Gonzalez-Robayna IJ, Falender AE, Ochsner S, Firestone GL, Richards JS. Follicle-stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-induced kinase (Sgk): evidence for A kinase-independent signaling by FSH in granulosa cells. Mol Endocrinol 2000; 14: 1283–1300.
39. Tsujimoto Y, Shimizu S. Bcl-2 family: life-or-death switch. FEBS Lett 2000; 466:6–10.
40. Pece S, Chiariello M, Murga C, Gutkind JS. Activation of the protein kinase Akt/PKB by the formation of E-cadherin-mediated cell-cell junctions. Evidence for the association of phosphatidylinositol 3-kinase with the E-cadherin adhesion complex. J Biol Chem 1999; 274: 19347–19351.
41. Carmeliet P, Lampugnani MG, Moons L, Breviario F, Compernolle V, Bono F, Balconi G, Spagnuolo R, Oostuyse B, Dewerchin M, Zanetti A, Angellilo A, Mattot V, Nuyens D, Lutgens E, Clotman F, de Ruiter MC, Gittenberger-de Groot A, Poelmann R, Lupu F, Herbert JM, Collen D, Dejana E. Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. Cell 1999; 98:147–157.
42. Machell NH, Blaschuk OW, Farookhi R. Developmental expression and distribution of N- and E-cadherin in the rat ovary. Biol Reprod 2001; (in press).
43. Peluso JJ, Pappalardo A, Trolice MP. N-Cadherin-mediated cell contact inhibits granulosa cell apoptosis in a progesterone-independent manner. Endocrinology 1996; 137:1196–1203.
44. Trolice MP, Pappalardo A, Peluso JJ. Basic fibroblast growth factor and N-cadherin maintain rat granulosa cell and ovarian surface epithelial cell viability by stimulating the tyrosine phosphorylation of the fibroblast growth factor receptors. Endocrinology 1997; 138:107–113.
45. Makrigiannakis A, Coukos G, Christofidou-Solomidou M, Gour BJ, Radice GL, Blaschuk O, Coutifaris C. N-Cadherin-mediated human granulosa cell adhesion prevents apoptosis—a role in follicular atresia and luteolysis? Am J Pathol 1999; 154:1391–1406.