Introduction

Ovaries undergo extensive tissue remodelling during the reproductive cycle, which is important for follicle growth and ovulation, as well as formation and regression of corpora lutea. This tissue remodelling is dependent on cell division, dissociation, migration and adhesive restructuring. Two major classes of adhesive receptors expressed by epithelial cells are cell adhesion molecules, which mediate cell–cell adhesion (Takeichi, 1991), and integrins, which mediate cell–extracellular matrix adhesion (Hynes, 1992). Both calcium-dependent (for example, cadherins; Farookhi and Blaschuk, 1991) and integrins, which mediate cell–extracellular matrix adhesion (Hynes, 1992). Both calcium-dependent (for example, cadherins; Farookhi and Blaschuk, 1991) and independent (for example, neural cell adhesion molecule (NCAM); Mayerhofer et al., 1991) forms of cell adhesion molecules have been identified in the ovaries and linked with cyclic remodelling. Epithelial cadherin (E-cadherin) is a member of the cadherin family of calcium-dependent cell adhesion molecules that is expressed in granulosa (Blaschuk and Farookhi, 1989; Ryan et al., 1996), luteal (Khan-Dawood et al., 1996 a,b) and surface epithelial (Hoffman et al., 1993; Inoue et al., 1992; Ryan et al., 1996) cells of the ovaries. In addition, E-cadherin and other members of the cadherin family of adhesion proteins are constituents of mammalian oocytes (Rufas et al., 2000). E-cadherins are transmembrane glycoproteins that mediate homotypic cell–cell adhesion and are important in the formation and maintenance of cell–cell contacts and junctions between epithelial cells (Gumbiner et al., 1988).

Although cadherin proteins have been identified in cells of developing follicles and corpora lutea, their role in ovarian function is unclear. There is evidence that cadherins contribute to the structural integrity of follicles and corpora lutea. For example, in atretic pig follicles the loss of granulosa cell adhesion and follicle wall integrity is associated with a significant decrease in the expression of E-cadherin by granulosa cells (Ryan et al., 1996). Likewise, a similar decrease in E-cadherin protein has been reported in regressing baboon corpora lutea during the late luteal phase of the reproductive cycle (Khan-Dawood et al., 1996 a,b). In addition to maintaining the structural framework of ovaries, cell–cell contact is critical for ovarian growth and
differentiation. Disturbances in ovarian cell adhesion in vitro are linked with increased atresia, cyst formation and insufficient luteal function (Khan-Dawood et al., 1996b; Makrigiannakis et al., 1999). In vitro studies indicate that cell–cell communication and aggregation are important for differentiation of rat granulosa cells by enhancing FSH induction of both aromatase and LH receptors (Farookhi and Desjardins, 1986). In rat granulosa cells, inhibiting N-cadherin activity in vitro disrupted cell–cell contact and promoted apoptosis (Peluso et al., 1996). However, even when N-cadherin activity was blocked, 30–40% of the granulosa cells still formed aggregates, which indicates that other cadherins or adhesion molecules are involved in maintaining adhesion of granulosa cells.

Given the epithelial nature of the ovaries, the present study focuses on the importance of E-cadherin activity in pig granulosa cells. E-cadherin activity in vitro was neutralized with specific antisera and the impact of neutralization on aggregation of granulosa cells, DNA synthesis and cell proliferation was investigated.

Materials and Methods

Materials

Human recombinant IGF-I was purchased from Bachem Bioscience Inc (King of Prussia, PA). [3H]-thymidine was purchased from ICN Biomedicals Inc (Costa Mesa, CA). Monoclonal anti-human E-cadherin antibody, specific for the cytoplasmic domain of E-cadherin (T-lab) was from Transduction Laboratories (Lexington, KY). Rat anti-mouse uvomorulin (clone DECMA-1 in rat ascites fluid), which cross-reacts with the extracellular domain of E-cadherin (Vestweber and Kemler, 1985) and cross-reacts with pig ovarian tissue (Ryan et al., 1996). Negative antibody controls included substitution of DECMA-1 in rat ascites fluid with normal mouse ascites fluid (Sigma) or with an antibody specific for the intracellular domain of E-cadherin (mouse anti-human E-cadherin (T-lab), which is inaccessible in intact cells in culture. This approach is based on a similar study of E-cadherin function in human trophoblast cells in which the effects of antisera directed against the extracellular and cytoplasmic domains of E-cadherin were compared (Coutifaris et al., 1991). One hour after addition of antibody or ascites fluid, fetal bovine serum (FBS; 10%) was added to facilitate growth and attachment of granulosa cells. The ability of granulosa cells to recover from the effects of E-cadherin immunoneutralization was also tested. Granulosa cells were exposed to E-cadherin antibody or ascites fluid for 24 h and then washed and incubated with either serum-containing TCM-199 only or TCM-199 containing fresh E-cadherin antibody or ascites fluid for an additional 24 h. At 48 h after plating, the effects of E-cadherin immunoneutralization on DNA synthesis, proliferation, adhesion and clustering of granulosa cells were evaluated.

Incorporation of [3H]thymidine and proliferation of granulosa cells

After 48 h incubation of granulosa cells with the E-cadherin antisera (DECMA-1 or mouse anti-human E-cadherin) or control ascites fluid, the media was removed, replaced with media containing 1.0% FBS and IGF-I (3 ng ml−1) and incubated for 24 h. [3H]thymidine (4 μCi ml−1) was added for the last 6–8 h of treatment. Substratum-attached cells were lifted from the wells with 0.25% (v/v) trypsin and EDTA (5 mmol l−1), collected onto glass fibre filters using a multiwell cell harvester (Cambridge Technology Inc, Watertown, MA) and incorporation of [3H]thymidine into DNA was assessed.

The impact of E-cadherin immunoneutralization on cell proliferation was monitored using a crystal violet solution to stain the nuclei of the granulosa cells as described by Kueng et al. (1989). In brief, substratum-attached granulosa cells were fixed in 1.1% (v/v) glutaraldehyde (final concentration) for 15 min at room temperature and washed in deionized water (3 × 5 min washes). The cells were stained with 100 μl 0.1% (w/v) crystal violet in PBS (0.015 mol l−1, pH 7.5) for

K. M. Kirkup et al.

Culture of granulosa cells

Ovaries from immature pigs were collected from a local abattoir. Granulosa cells from medium-sized (3–5 mm in diameter) pig follicles were isolated by follicle puncture as described by Ohleth and Bagnell (1995) and suspended in TCM-199 supplemented with 100 U penicillin ml−1, 100 μg streptomycin ml−1, 10 μg gentamicin ml−1, and 0.5 μg fungizone ml−1. Granulosa cells were incubated with 2 mg collagenase ml−1 and 10 μg DNase I ml−1 for 10–20 min at 37°C with intermittent Pasteur pipetting to aid dispersion of granulosa cells, filtered over a 177 μm mesh, washed twice with TCM-199 and plated. For incorporation of [3H]thymidine and cell proliferation assays, granulosa cells (100 000 cells per well; at least four wells per treatment) were inoculated into 96-well plates in 200 μl TCM199. Granulosa cells (100 000 cells per 200 μl TCM-199) were plated in fibronectin-coated (40 μg ml−1) Labtek eight-chamber slides (Nalge Nunc International, Naperville, IL) to observe granulosa cell nuclei using hydroethidine.

Immunoneutralization

Granulosa cells were exposed to an anti-E-cadherin antibody specific for the extracellular domain of E-cadherin (rat anti-mouse uvomorulin, clone DECMA-1 in rat ascites fluid) at the time of plating (0 h) to evaluate the impact of E-cadherin immunoneutralization on DNA synthesis, proliferation, adhesion and clustering of granulosa cells. This E-cadherin antisera neutralizes E-cadherin activity, dissociates Madin–Darby canine kidney (MDCK) cells (Vestweber and Kemler, 1985) and cross-reacts with pig ovarian tissue (Ryan et al., 1996). Negative antibody controls included substitution of DECMA-1 in rat ascites fluid with normal mouse ascites fluid (Sigma) or with an antibody specific for the intracellular domain of E-cadherin (mouse anti-human E-cadherin (T-lab), which is inaccessible in intact cells in culture. This approach is based on a similar study of E-cadherin function in human trophoblast cells in which the effects of antisera directed against the extracellular and cytoplasmic domains of E-cadherin were compared (Coutifaris et al., 1991). One hour after addition of antibody or ascites fluid, fetal bovine serum (FBS; 10%) was added to facilitate growth and attachment of granulosa cells. The ability of granulosa cells to recover from the effects of E-cadherin immunoneutralization was also tested. Granulosa cells were exposed to E-cadherin antibody or ascites fluid for 24 h and then washed and incubated with either serum-containing TCM-199 only or TCM-199 containing fresh E-cadherin antibody or ascites fluid for an additional 24 h. At 48 h after plating, the effects of E-cadherin immunoneutralization on DNA synthesis, proliferation, adhesion and clustering of granulosa cells were evaluated.

Incorporation of [3H]thymidine and proliferation of granulosa cells

After 48 h incubation of granulosa cells with the E-cadherin antisera (DECMA-1 or mouse anti-human E-cadherin) or control ascites fluid, the media was removed, replaced with media containing 1.0% FBS and IGF-I (3 ng ml−1) and incubated for 24 h. [3H]thymidine (4 μCi ml−1) was added for the last 6–8 h of treatment. Substratum-attached cells were lifted from the wells with 0.25% (v/v) trypsin and EDTA (5 mmol l−1), collected onto glass fibre filters using a multiwell cell harvester (Cambridge Technology Inc, Watertown, MA) and incorporation of [3H]thymidine into DNA was assessed.

The impact of E-cadherin immunoneutralization on cell proliferation was monitored using a crystal violet solution to stain the nuclei of the granulosa cells as described by Kueng et al. (1989). In brief, substratum-attached granulosa cells were fixed in 1.1% (v/v) glutaraldehyde (final concentration) for 15 min at room temperature and washed in deionized water (3 × 5 min washes). The cells were stained with 100 μl 0.1% (w/v) crystal violet in PBS (0.015 mol l−1, pH 7.5) for
20 min at room temperature. Excess dye was removed by washing with deionized water, the cells were dried and bound dye was solubilized in 100 μl 10% (v/v) acetic acid. The absorbance of the dye extracts was measured at 590 nm using a Biotek EL340 microplate reader (Winooski, VT). A standard curve was generated for each experiment by inoculating wells in quadruplicate with increasing numbers of granulosa cells (0–300,000 cells per well).

**Visualization of cell nuclei for monitoring cell adhesion and clustering**

After immunoneutralization of E-cadherin, the nuclei of granulosa cells were visualized as described by Bucana et al. (1986), with some modifications. Substratum-attached cells were washed with Hank’s basic salt solution (HBSS) containing 1 mmol l−1 calcium l−1 (3 × 2 min washes) and stained with 14 μg hydroethidine ml−1 for 15–30 min at room temperature in the dark. After removal of the hydroethidine, the granulosa cells were fixed in 4.0% formaldehyde in PBS temperature in the dark. After removal of the hydroethidine, the granulosa cells were incubated for 5 min and mounted using N-propyl gallate. Cells were viewed and photographed at a wavelength of 535/585 nm using a fluorescent microscope (Zeiss Axioskop). Cells were scored visually by two observers on a scale of 1–5 for three parameters: cluster number, cluster size and cell-cell proximity within the clusters. A cell cluster was defined as a group of > ten cells in cell-cell contact. The number of clusters per well was quantified with a rating of 1 for a small number of clusters and 5 indicating a large number of clusters per well was quantified with a rating of 1 indicating very small clusters. Relative cluster size was estimated from very small clusters (10–15 cells per cluster; rating = 1) to very large (> 150 cells per cluster; rating = 5). Cell-cell proximity scores were based on the degree of cell-cell contact and relative distance between adjacent nuclei within each cluster. A score of 1 signified relatively little cell-cell contact within a cluster, whereas a score of 5 indicated a high degree of compaction within a cluster such that most adjacent cells were in direct contact with each other. Granulosa cells incubated in 10% serum-containing media were used as positive controls for adhesion and clustering experiments. The negative control consisted of granulosa cells incubated in serum-free media, which causes cells to undergo apoptosis.

**Statistical analysis**

The results are presented as mean ± SEM of at least three experiments using independent pools of granulosa cells. Thymidine incorporation data are presented as percentage relative to control (cells only), with the control equal to 100%. Data were analysed by analysis of variance and tested for differences using Fisher’s least significance difference test on Statview 4.05 application (Abacus Concepts, Berkeley, CA). The level of significance was P < 0.05.

![Fig. 1. Effect of E-cadherin antibody on DNA synthesis and proliferation of granulosa cells](image)
was therefore inaccessible to intact cells in culture, there was no effect on DNA synthesis in granulosa cells.

On the basis of the thymidine incorporation studies, an anti-uvomorulin E-cadherin antiserum (DECMA-1) concentration of 1:200, which resulted in 50% inhibition in DNA synthesis, was used to study the effects of E-cadherin immunoneutralization on proliferation, adhesion and clustering of granulosa cells. When granulosa cells were incubated continuously with the anti-uvomorulin E-cadherin antibody for 48 h (Fig. 2a), there was a 28% decrease in the number of cells, which was a significant decrease ($93.0 \pm 6.7 \times 10^3$) compared with granulosa cells incubated in serum-containing media only ($129.0 \pm 5.9 \times 10^3$). In contrast, exposure to either mouse ascites fluid or the anti-E-cadherin antibody directed against the cytoplasmic domain did not alter cell proliferation when compared with the control. Under serum-free conditions, the number of granulosa cells was reduced by 90.7% compared with cells in serum-containing media only. Furthermore, incubation of granulosa cells with the anti-uvomorulin E-cadherin antibody for 24 h, followed by either antibody removal or replenishment for 24 h did not alter the inhibitory effect of the anti-uvomorulin antibody on the number of granulosa cells (Fig. 2b).

Effect of E-cadherin antibody on cell–cell association parameters in pig granulosa cells

The impact of E-cadherin immunoneutralization on cell–cell association of granulosa cells in culture is shown (Fig. 3). After exposure to the anti-uvomorulin antibody for 24 h (Fig. 3b) the size of the cell clusters was decreased, whereas the distances between cells within the clusters were increased compared with granulosa cells incubated with control mouse ascites fluid (Fig. 3a). Overall, the control granulosa cells formed larger clusters with more cells packed tightly together than did those incubated with E-cadherin antibody. A representative very large cluster (score = 5) is shown (Fig. 3a). Although granulosa cells attached to the substratum in the presence of DECMA-1, the primary effect of E-cadherin neutralization was a reduction in cell–cell contact. Three representative clusters of granulosa cells are shown (Fig. 3b: medium and small clusters), illustrating the reduction in cluster size and cell–cell contact in the presence of DECMA-1 antiserum. These morphological responses to E-cadherin immunoneutralization were quantified (Fig. 4). A significant decrease in the cluster size of granulosa cells without any change in cluster number compared with control granulosa cells incubated with mouse ascites fluid was observed in granulosa cells incubated with the anti-uvomorulin E-cadherin antibody for 48 h. In addition, the high degree of cell–cell contact observed in control granulosa cells was decreased significantly in granulosa cells exposed to the E-cadherin antibody. These effects of E-cadherin immunoneutralization on associations among granulosa cells could not be reversed by removal of the antibody after 24 h and incubation in antibody-free media for an additional 24 h (data not shown). Likewise, addition of fresh E-cadherin antibody after the initial 24 h exposure failed to significantly change any of the cell–cell association parameters measured at 48 h.

**Fig. 2.** Effect of E-cadherin immunoneutralization on proliferation of pig granulosa cells *in vitro*. (a) After 48 h in the presence or absence of anti-E-cadherin antibody (uvomorulin [■] or mouse anti-human E-cadherin [□]: T-lab: 1:200) or mouse ascites fluid ([□]: 1:200), granulosa cells were stained with a crystal violet solution and absorbance was measured as a function of cell number. (b) Granulosa cells were incubated with the E-cadherin antibody for 24 h, followed by either antibody removal or replenishment for an additional 24 h. Additional controls included cells only (□) in serum-containing media and cells incubated in serum-free media (□). Data are mean ± SEM ($n > 3$). Values in the same panel with different letters are significantly different ($P < 0.05$).
Discussion

Although the presence of E-cadherin has been documented in a variety of reproductive tissues, the functional role that E-cadherin plays in reproductive tissue growth and remodelling has not been elucidated fully. In the present study, treatment of pig granulosa cells with an anti-E-cadherin antibody, DECMA-1, directed against the extracellular domain of E-cadherin, inhibited IGF-I-stimulated DNA synthesis in a dose-dependent fashion. In addition, loss of E-cadherin activity significantly inhibited proliferation of granulosa cells, reduced cell–cell contact and decreased the ability of granulosa cells to form aggregates in vitro. These data are similar to those reported for luteal cells in culture, as immunoneutralization of E-cadherin activity induces a similar disruption in cell–cell contact in cells from early luteal phase corpora lutea (Khan-Dawood et al., 1996b). Taken together, these findings are consistent with the concept that E-cadherin-mediated adhesion is important for maintaining the structural integrity of follicles and corpora lutea.

E-cadherin is a transmembrane protein that mediates cell–cell adhesion via homophilic binding of the N-terminal extracellular domain present on adjacent cells (Takeichi, 1991). Intercellularly, the cytoplasmic domain of E-cadherin interacts with proteins, α-, β- and γ-catenins, that link cadherin molecules to the actin cytoskeleton (Ranscht, 1994). In the present study, E-cadherin activity was immunoneutralized using an antibody specific for the extracellular domain of E-cadherin, DECMA-1. In other systems, this antibody is reported to block aggregation of mouse embryonic carcinoma cells and compaction of pre-implantation embryos, and disrupts confluent cultures of MDCK epithelial cells (Vestweber and Kemler, 1985). The inhibition of growth of granulosa cells in the presence of the DECMA-1 antibody was specific, as shown by the lack of effect when granulosa cells were incubated with another anti-E-cadherin antibody specific for the cytoplasmic domain of E-cadherin, which was therefore inaccessible in intact granulosa cells. The same approach was taken by Coutifaris et al. (1991), who demonstrated that syncytium formation in human trophoblast cells in culture was inhibited in the presence of an antiserum directed against the extracellular domain of E-cadherin. However, another antiserum directed against the cytoplasmic domain of E-cadherin had no effect on the adhesion of these trophoblast cells, thus demonstrating the importance of the extracellular domain for adhesion.

In preliminary studies it was observed that once granulosa cells were attached to the plate and in contact with each other (48 h after plating in serum) addition of the DECMA-1...
antibody did not significantly influence cell growth or cell–cell association. However, targeting the extracellular domain of E-cadherin with the antibody before association of granulosa cells in culture was most effective in disrupting granulosa cell function. These data indicate that there was greater access for the antibody to bind to the extracellular domain of E-cadherin in dispersed granulosa cells. Likewise, in the highly confluent MDCK cell line, binding of the DECA-M antibody could be detected only after detergent permeabilization of fixed cells, which presumably allows the antibody to bypass tight junctional barriers between cells in contact (Vestweber and Weber, 1985). In the present study, it was also found that when granulosa cells were exposed to the E-cadherin-neutralizing antibody, cluster size decreased, whereas cluster number was not changed significantly. In addition, adhesion of peripheral granulosa cells in a cluster decreased, whereas cell–cell distance increased. Vestweber and Kemler (1985) reported a similar pattern of adhesive disruption in MDCK cells after DECA-M-induced E-cadherin immunoneutralization. Vestweber and Kemler (1985) found that DECA-M bound only to the periphery of fixed, unpermeabilized confluent MDCK cells. This finding was consistent with their observation that DECA-M dissociated MDCK cells at the periphery of groups of cells in contact and progressively disrupted cell–cell adhesion in confluent areas. As reported for MDCK cells (Vestweber and Kemler, 1985), no evidence for an effect of DECA-M on adhesion of granulosa cells to the substratum was found in the present study. Taken together, these data indicate the importance of a functional extracellular E-cadherin domain in facilitating cell aggregation and cell–cell adhesion.

Our observation that immunoneutralization with the DECA-M antibody only blocked growth and cell–cell association of granulosa cells partially supports the contention that, like most other cells, granulosa cells express other adhesion molecules, including other types of cadherins. For example, granulosa cells of growing pre-antral and antral follicles express both neural cell adhesion molecule (Mayerhofer et al., 1991) and N-cadherin (Makrigiannakis et al., 1999). However, Peluso et al. (1996) demonstrated that loss of N-cadherin function inhibited aggregation of rat granulosa cells only partially. Likewise, in the present study, using an E-cadherin-function-disturbing antibody, only partial inhibition of DNA synthesis and cell clustering by granulosa cells was observed, even when high concentrations of antisera were used. Furthermore, the observation that growth and cell–cell association of granulosa cells were not affected further by longer incubation in the presence of fresh E-cadherin antisera supports the concept that redundancy in expression of adhesion proteins by granulosa cells is involved in ensuring that cell–cell contact is maintained.

The importance of cell–cell contact for function and survival of granulosa cells is well documented. Contact between granulosa cells is necessary for FSH-stimulated LH receptor induction and aromatase activity (Farookhi and Desjardins, 1986). Single granulosa cells in culture are twice as likely to be apoptotic compared with aggregated granulosa cells (Luciano et al., 1994). In vitro studies demonstrate that blocking N-cadherin function by immunoneutralization increases apoptosis in both rat (Peluso et al., 1996) and human (Makrigiannakis et al., 1999) granulosa cells. Although apoptosis of pig granulosa cells was not monitored directly in the present study, a reduction in the number of hydroethidine-stained granulosa cells within cell clusters was observed in the presence of the E-cadherin antibody. These data, together with the decreases in granulosa cell numbers and DNA indicate that disrupting E-cadherin-mediated cell adhesion is detrimental to the viability of pig granulosa cells. This finding is supported further by our observation that when the E-cadherin antibody was removed from the cultures, there was no evidence for recovery of the growth of granulosa cells or in their ability to form cell clusters.

The cell dynamics investigated in the present study are important to our understanding of the function of cadherins in reproductive tissues, such as the ovary. Extensive remodelling and alterations in tissue adhesiveness occur in the female reproductive system on a cyclical basis. The data presented here support the contention that cadherin-mediated adhesion is important for both the structure and function of reproductive tissues (for review see Rowlands et al., 2000). Specifically, these studies emphasize that E-cadherin is necessary for maintaining contact and cell proliferation of granulosa cells in vitro. The results of the present study, together with the evidence that in atretic follicles (Ryan et al., 1996) and regressing corpora lutea (Khan-Dawood et al., 1996 a,b) E-cadherin expression is lost, indicate that E-cadherin plays a fundamental role in the dynamic changes in cell–cell contact that occur on a cyclical basis in the ovaries. Given the importance of granulosa cell adhesion in maintaining follicular integrity and promoting growth and survival of granulosa cells, regulation of ovarian E-cadherin expression warrants further study.

The authors would like to acknowledge the assistance of K. Ohlth and J. Lenhart, and Leidy’s Inc for the supply of pig ovaries. This research was supported by USDA (93–37203–8979), the CH Cook Undergraduate Honors Program and the NJ Agricultural Experiment Station (NJ 06136).

References


Farookhi R and Desjardins J (1986) Luteinizing hormone receptor induction in dispersed granulosa cells requires estrogen Molecular and Cellular Endocrinology 47 13–24


model of ovarian epithelial carcinogenesis: changes in cell–cell communication and adhesion occurring during neoplastic progressionregression.


Kueng W, Silber E and Eppenberger U (1989) Quantification of cells cultures on a 96-well plate Analytical Biochemistry 182 16–19


Takeichi M (1991) Cadherin cell adhesion receptors as a morphogenetic regulator Science 22 1451–1455