Immunocytochemical localization and expression of E-cadherin and β-catenin in the human corpus luteum

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We have previously shown that the protein connexin-43 which forms the connexons in gap junctions is present in the human corpus luteum. Abundant expression of connexin-43 is seen in the mid-luteal phase corpora lutea. Since the formation of gap junctions in a tissue requires the presence of adherens junctions formed by the cadherins, our aim in these studies was firstly to localize immunocytochemically E-cadherin and β-catenin (a cytoplasmic protein associated with E-cadherin) in the human corpus luteum, and secondly to determine the concentrations of these proteins in the early, mid- and late luteal phase human corpora lutea. E-cadherin was localized to the periphery of luteal cells and was not detected in non-luteal tissue. β-catenin was observed in the cytoplasm of the luteal cells. Abundant expression of E-cadherin was observed by Western analysis in the early luteal phase and the level of expression was significantly different from that observed in the mid- and late luteal phase corpora lutea. In contrast the concentrations of β-catenin were higher in the mid-luteal phase compared to the early luteal phase. The differential expression of the cell adhesion molecule E-cadherin suggests that it may play a significant role in cell-to-cell communication in the corpus luteum, and in the cyclic development and demise of this tissue.

Key words: β-catenin/corpus luteum/E-cadherin/human/ovary

Introduction

After successful rupture of the ovarian follicle and release of the ovum, the residual cellular components of the follicle form the corpus luteum. Progesterone, the major secretory product of these cells in the human, is synthesized and secreted by the steroidogenic cells of this tissue. We have recently demonstrated the presence of gap junctions and the associated protein connexin-43 in the human and baboon corpus luteum of the menstrual cycle. The quantification of the protein during different stages of luteal development suggests that translation of the protein may be regulated by endocrine or paracrine factors (Khan-Dawood et al., 1996). In addition to connexins, it has been suggested that the group of cell adhesion molecules (CAMs) the cadherins, a family of calcium dependent cell surface glycoproteins, are required for intercellular communication via gap junctions (Musil et al., 1990; Meyer et al., 1992). In differentiated tissues, the cadherin–catenin complexes are localized to intermediate or adherens junctions (Boller et al., 1985; Volk and Geiger, 1986). These adhesion junctions are prerequisites for the formation not only of gap junctions but are needed for the organization of the cytoskeleton of the cell (Nelson, 1992).

The cadherins are single pass transmembrane proteins which are conformationally stable in the presence of extracellular calcium. The calcium binding and adhesion recognition sites are critical for their function (Geiger and Ayalon, 1992). The cytoplasmic domains also include areas that are critical for interaction with the cytoskeleton of the cell (Hirano et al., 1987). Within the cytoplasm the cadherins associate tightly with a number of different cytoplasmic proteins. These include the catenins (α-, β-, and γ-) which bind to the conserved cytoplasmic region of the E-cadherin molecule (Ozawa et al., 1989; Magee and Buxton, 1991). β-Catenin is a 92 kDa protein which may link the cadherins to the actin-based cytoskeleton. This interactive binding to the cadherins is an absolute requirement since deletions in the cytoplasmic domain of E-cadherin that eliminate catenin binding result in a loss of adhesion (Ozawa et al., 1990).

In human ovaries, E-cadherin has been localized immunocytochemically to ovarian tumour tissues, and in the normal ovary, only the germinal epithelium was shown to be E-cadherin positive in tissues examined without a corpus luteum (Inoue et al., 1992). Thus, no information is available on the presence or absence of adhesion proteins in the adult human ovary which is undergoing normal cycles or in the corpus luteum. Our hypothesis is that, following ovulation, restructuring of the remaining tissue which forms the corpus luteum requires 'cell adhesion'. Therefore our aim was to: (i) localize E-cadherin and β-catenin in the corpus luteum; and (ii) examine the concentrations of E-cadherin and β-catenin in the three luteal phases of corpora luteal development, early, mid- and late.

Materials and methods

Tissues

Human luteal tissues were obtained from pre-menopausal patients (aged 24–35 years) with a history of regular menstrual cycles and
undergoing surgery for non-endocrine gynaecological indications as previously described (Dawood and Khan-Dawood, 1986). Tissues were grouped as follows with n = 5 in each group: days 14–19 (early luteal phase); days 20–25 (mid-luteal phase); and days 26–30 (late luteal phase) of the menstrual cycle. Positive control tissue (human adenocarcinoma) was obtained from a 42 year old female undergoing total abdominal hysterectomy and bilateral salpingo-oophorectomy. Collection of these tissues was approved by the Institutional Review Board for Human Research at the University of Texas Health Science Center at Houston, Texas, USA. Each corpus luteum was cut in half and was either frozen immediately in liquid nitrogen and stored at −80°C until processed for Western analysis, or fixed in Bouin’s solution (Sigma Chemical Co., St. Louis, MO, USA) for 24 h at 22°C.

Antibodies
A highly specific monoclonal antibody to human E-cadherin (HECD-1, Lot No. 002FD) was supplied by Takara Biochemical Inc., (Berkeley, CA, USA) and Zymed Laboratories Inc., (San Francisco, CA, USA). This antibody was obtained by immunizing against a human breast tumour cell line, MCF-7, followed by purification with ammonium sulphate precipitation and anion exchange chromatography. The antibody has been demonstrated to be specific for E-cadherin, reacts strongly with human epithelial cadherin and recognizes a band at 120 kDa (Shimoyama et al., 1989a). The positive control used in the Western analyses was a mouse macrophage cell lysate (Transduction Laboratories, Lexington, KY, USA). The monoclonal anti-β-catenin antibody, raised against a 23 kDa protein fragment corresponding to residues 571–781 of mouse β-catenin, was obtained from Transduction Laboratories. The antibody was purified from mouse ascites using chromatographic techniques. The positive control used in the Western analyses for β-catenin was a human endothelial cell lysate (Transduction Laboratories). The secondary antibody, polyclonal anti-mouse, was developed in goat and immunosorbent to both immobilized human immunoglobulin (IgG) and mouse IgG (Transduction Laboratories).

Immunocytochemistry
After fixation each tissue was sequentially dehydrated and embedded in Surgipath paraffin (Surgipath Medical Industries Inc., Grayslake, IL, USA) as previously described (Khan-Dawood, 1987). Tissue sections of 4–5 μm were deparaffinized, washed and treated with 3% hydrogen peroxide to block endogenous peroxidases. Non-specific effects were blocked by treatment of the tissue with 2% normal goat serum in phosphate buffered saline for 60 min at room temperature. The primary antibodies for E-cadherin and β-catenin were used at dilutions of 1:100. The antigen-antibody complex was detected using the Renaissance chemiluminescence reagent according to the manufacturer’s protocol (DuPont NEN, Boston, MA, USA).

Statistical analysis
The Western blots of five different samples were analysed. Areas corresponding to the proteins were quantified by determining individual band intensities on a Phosphorimagery System 400 (Molecular Dynamics, Sunnyvale, CA, USA) using Image Quant 3.2 software. The densitometric values obtained were statistically analysed using a one-way analysis of variance to compare samples from each stage of luteal development. Significant differences between the three developmental phases were determined using the multiple range companion procedure of the Newman–Keuls test. Differences were considered to be significant at P <0.001. For E-cadherin, bands at 120 kDa and for β-catenin bands at 92 kDa were analysed.

Results
E-cadherin immunoreactivity observed as intense staining at the periphery of the luteal cells was seen in the corpus luteum; positive immunoreactivity was not observed in non-luteal tissue (Figure 1a). β-catenin was also localized to the luteal cells; however, in contrast the positive immunoreactivity was present in the cytoplasm of the cells (Figure 1b). E-cadherin was also localized to human adenocarcinoma tissue (Inoue et al., 1992) which was used as a positive control (Figure 1c). In the absence of the primary antibody, no immunoreactivity was observed in the corpora lutea (Figure 1d) and neither was immunoreactivity observed in the cardiac tissue confirming previous data (Figure 1e) (Shimoyama et al., 1989b).

Western analysis
The presence and concentrations of E-cadherin in corpora lutea from each stage of luteal development were determined by Western analysis of the membrane fraction (Khan-Dawood et al., 1996). Briefly, the frozen tissue was homogenized in five volumes of sucrose-3-N-morpholino-propanesulphonic acid buffer (MOPS; Sigma Chemical Co.) pH 7.2 using a polytron P-10 homogenizer. The buffer consisted of 250 mM MOPS, 20 mM sucrose, 1 mM phenylmethylsulphonyl fluoride, 2 mM dithiothreitol, 2 mM CaCl₂ (Tohma et al., 1987) 20 μg/ml trypsin inhibitor, 2 μg/ml antipain, 10 μg/ml benzamidine, 1 μg/ml chymostatin and 1 μg/ml pepstatin A. Following centrifugation at 1000 g for 10 min, the supernatant was recentrifuged at 100 000 g for 60 min. The supernatant soluble fraction was used to analyse the cytoplasmic protein β-catenin. The pellet obtained was resuspended in MOPS buffer and recentrifuged at 100 000 g for 30 min. The membrane fraction thus obtained was used for E-cadherin analysis. Protein was determined in each tissue fraction (Lowry et al., 1954). Cardiac tissue was similarly processed. Electrophoresis of each sample (10 μg protein) was performed according to Laemmli (1970). Samples were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) with a 7% separating gel and a 4% stacking gel. Molecular weight markers used were pre-stained SDS–PAGE standards (Bio-Rad, Richmond, CA, USA). Following transfer onto nitrocellulose membranes (Hybond-C Super; Amersham, Arlington, IL, USA), the membrane were treated with 5% dry milk in phosphate-buffered saline for 60 min at room temperature and incubated with the primary antibodies at the following dilutions: E-cadherin (1:5000) and β-catenin (1:1000) for 16 h at 4°C. The antigen–antibody complex was detected using the Renaissance chemiluminescence reagent according to the manufacturer’s protocol (Dupont NEN, Boston, MA, USA).
Figure 1. Immunocytochemical localization of (a) E-cadherin and (b) β-catenin in an early corpus luteum; (c) localization of E-cadherin in an ovarian adenocarcinoma used as a positive control; (d) section of a corpus luteum which was treated similarly to (a) and (b), however the primary antibody was replaced with preimmune mouse serum. Absence of immunoreactive positive reaction is evident. (e) Cardiac tissue used as a tissue negative control. Positive immunoreactivity is absent. Original magnification X40. Bar = 10 μm in a, c, d, and e. Bar = 20 μm in b.
The expression and role of E-cadherin in corpora lutea have not been previously examined. In the rat granulosa cells E-cadherin is expressed and appears to be regulated by the local hormonal environment, thus oestradiol was shown to increase cadherin expression by granulosa cells (Blaschuk and Farookhi, 1989; Farokhi and Blaschuk, 1989). Other adhesion molecules such as the neural cell adhesion molecule (NCAM) have been demonstrated in the endocrine cells of the adult rat (Langley et al., 1996). Interest-
egly, examination of concentrations of connexin-43 indicated abundant levels in corpora lutea during the mid-luteal phase. Thus the early luteal phase E-cadherin concentrations, together with mid-luteal phase connexin 43 concentrations suggests that rapid cell–cell adhesion may be necessary following ovulation for maximal intercellular communication to occur between the luteal cells when the synthesis of progesterone is accelerated.

In general, increased cadherin expression causes tighter association of cells and if the activity of cadherins is suppressed, cells forming tissues tend to dissociate (McNeill et al., 1990). In this study we found cadherin expression in the late luteal phase to be comparatively low. As previously shown, connexin-43 expression is also low during this stage of the luteal phase (Khan-Dawood et al., 1996). In the absence of pregnancy and the need for maintaining the corpus luteum, the process of luteolysis begins and therefore, the cells of the corpus luteum may not need to communicate to the same extent as in the early and mid-luteal phases.

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(Mayerhofer et al., 1991). More recently it has been suggested that the human granulosa cells express NCAM during in-vitro luteinization (Mayerhofer et al., 1994).

In summary, we have shown the presence of the cell adhesion molecule E-cadherin and E-cadherin-associated protein α-catenin in human corpora lutea, suggesting the presence of adherens junctions. Examination of the expression of these proteins in the various luteal phases indicates a differential expression of the peptides. Together with our data on connexin-43, we suggest that intercellular communication in the corpus luteum is an important factor in the overall production of progesterone. Decrease in the communicative activity may facilitate apoptosis (Trosko and Goodman, 1994) leading to luteolysis, unless signals emanating from the trophoblast-embryo interaction prevent this effect. This could result from the following sequence of events. In the late luteal phase we have observed that both connexin-43 and E-cadherin are down-regulated, suggesting that communication at gap junctions between cells is probably either absent or low. In the event that apoptosis-preventing signals may cross from cell to cell via gap junctions, this would no longer occur, and the activity of apoptosis-activating factors would become dominant, leading to an increase in cell death unless the tissue were rescued by human chorionic gonadotrophin (HCG) arising from the developing conceptus.

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