Cell junctional proteins in the human corpus luteum: changes during the normal cycle and after HCG treatment

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BACKGROUND: Regulation of tissue remodelling and ovarian permeability by intercellular adhesion complexes may be involved in normal and pathological ovarian function. Therefore, the occurrence, distribution and hormonal control of the adherens junction protein vascular endothelial cadherin (VE-cadherin) and the tight junction proteins occludin and claudin in the human corpus luteum (CL) were investigated. METHODS: CLs from patients undergoing hysterectomy for benign reasons were enucleated during early, mid- and late stages of the functional luteal phase and after HCG rescue in vivo. Immunostaining for occludin, claudins 1 and 5 and VE-cadherin was carried out on fixed tissue. Endothelial cells, granulosa lutein cells and theca lutein cells were identified by reference to serial sections immunostained for CD34, 17α-hydroxylase and 3β-hydroxy-steroid-dehydrogenase. Quantitative analyses were performed using image analyses. RESULTS: Occludin was localized to the plasma membrane of granulosa lutein cells and endothelial cells but was absent in theca lutein cells. Claudin 1 was exclusively localized to the plasma membrane of steroidogenic cells. Claudin 5 and VE-cadherin were only present in endothelial cells. After HCG administration in vivo, adherens and tight junction proteins were significantly down-regulated (P < 0.05). CONCLUSIONS: The decrease of junctional proteins after HCG treatment suggests a hormonal control of tight and adherens junctions in the CL associated with tissue remodelling and an increase in luteal permeability during early pregnancy.

Key words: adherens junctions/corpus luteum/junction proteins/permeability/tight junctions

Introduction

The human corpus luteum (CL) is a temporarily active endocrine gland consisting of mainly granulosa lutein cells, theca lutein cells and endothelial cells. These different cell types act synergistically to produce and release hormones. Intercellular junctions mediate adhesion, communication and permeability between the adjoining steroidogenic and endothelial cells (Bazzoni and Dejana, 2004; Walz et al., 2005). Major components of intercellular junctions are adherens and tight junctions (Dejana, 2004; Schneeberger and Lynch, 2004). These junctions are formed by different proteins but have common features. In both, adhesion is mediated by transmembrane proteins promoting homophilic interaction. The cytoplasmic domain of the transmembrane adhesion molecules binds to linker proteins which in turn anchor the adhesion complex to the cytoskeleton. Tight junctions, or zonula occludens, are the most apical components of the intercellular junctional complex, which completely seal the space between neighbouring cells. Thus, they are the most important structural complex to form a barrier to the diffusion of molecules from the vessel lumen to the tissue parenchyma (barrier function) and restrict the diffusion of molecules and water between the apical and basolateral plasma membranes (fence function) (Matter and Balda, 2003; Harhaj and Antonetti, 2004). There are different types of transmembrane proteins such as occludin and the various members of the claudin family, principally claudin 5 which is endothelial cell specific (Morita et al., 1999; Morita et al., 2003).

Adherens junctions play an important role in contact inhibition of endothelial growth and paracellular permeability (Bazzoni and Dejana, 2004). The most prominent adhesion molecule of endothelial adherens junctions is vascular endothelial cadherin (VE-cadherin), which has been shown to be directly involved in the maintenance of endothelial intercellular contacts both in vitro and in vivo (Dejana et al., 2000; Vestweber, 2000; Dejana, 2004).

The junctional proteins E-cadherin, β-catenin and zona occludens-1 (ZO-1) have been identified in the baboon CL (Khan-Dawood et al., 1996b). E-cadherin was found to have a cycle-dependent expression, and it was proposed that such proteins may play a role in the cyclic development and demise of the CL (Khan-Dawood et al., 1996a). Despite these observations, little is known about the specific expression and regulation of junctional proteins in the CL of any species and whether the regulation of junctional proteins in the CL is hormonally...
controlled. The cyclic growth and development of the CL takes place under the influence of gonadotrophins. Within the normal cycle, under the influence of LH, the lifespan of the CL is restricted to 14 days, whereas if pregnancy occurs, the CL is rescued by HCG and survives for several months. In the human CL, it was shown that luteal rescue is associated with the expansion of the luteal vasculature (Wulff et al., 2001a). It may be hypothesized that these remodelling processes may involve rearrangement of intercellular contacts and thus junctional proteins.

Here, the spatial and temporal expression and distribution of the junctional proteins occludin, claudins 1 and 5 and VE-cadherin were investigated for the first time in the human CL. It was hypothesized that the expression and distribution may change during the tissue remodelling seen during the normal luteal phase and especially after luteal rescue with HCG. It has been suggested that the endothocrine function of the CL, especially during pregnancy, is dependent on a permeable vasculature ensuring the feasibility of the CL to rapidly synthesize and release high amounts of hormones. We therefore investigated whether HCG could change the junctional proteins in the luteal vasculature.

Methods

Tissue collection and preparation

Corpora lutea from patients undergoing hysterectomy for benign reasons were enucleated during early (n = 6), mid- (n = 6) and late luteal phase (n = 6) and after HCG rescue (n = 4) as described in detail previously (Duncan et al., 1996; Rodger et al., 1997). The date of the pre-ovulatory LH surge was determined by measuring LH concentrations in serial early morning urine samples: LH + 1 to LH + 5 days = early, LH + 6 to LH + 10 = mid- and LH + 11 to LH + 14 = late luteal phase. Four women were administered i.m. injections of HCG (Profasi, Serono Laboratories, Welwyn Garden City, UK) from LH + 7 in daily doubling doses, starting at 125 IU and continuing for 6–8 days to reproduce the hormonal changes of early pregnancy (Illingworth et al., 1990). The tissue was fixed in 4% paraformaldehyde for 24 h. In all cases, morphological dating of the luteal phase endometrium was used to confirm the luteal-phase classification. The study was approved by the Southeast of Scotland Medical Research Ethics Committee, and informed consent was obtained from all patients before tissue collection.

Immunohistochemistry

To distinguish between different compartments using methods that have been described previously (Fraser et al., 2005a), we stained consecutive sections for 17α-hydroxylase to identify theca cells, for 3β-hydroxy-steroid-dehydrogenase (3β-HSD) to detect steroidogenic cells (Figure 1) and for CD34 to localize endothelial cells. Further consecutive sections were stained for proteins of interest, namely occludin, claudins 1 and 5 and VE-cadherin. The following antibodies were used: rabbit anti-pig 17α-hydroxylase (CYP17, gift from Prof. I. Mason, University of Edinburgh, 1.750 dilution), rabbit anti-human 3β-HSD (gift from Prof. I. Mason, 1:1000 dilution), mouse anti-human CD34 (Serotec MCAP547, 1:100 dilution), rabbit anti-human occludin (Zymed, 71–1500; 1:100 dilution), rabbit anti-human claudin 1 (Zymed, 51–9000; 1:100 dilution), mouse anti-human claudin 5 (Zymed, 18–7364; 1:100 dilution) and mouse anti-human VE-cadherin (Chemicon MAB 1989; 1:20 dilution).

Sections of 5 μm were cut onto Tespa-coated slides. Sections were dewaxed in xylene and rehydrated in descending concentrations of ethanol. Negative controls were performed for all antibodies by replacing the first antibody with Tris-buffered saline (TBS). Antigen retrieval was achieved by pressure-cooking (Clypso pressure cooker, Tefal, Essex, UK) in 0.01 mol/l citrate buffer, pH 6 for 7 min. Sections were left for 20 min in hot buffer before cooling in TBS.

Immunohistochemical staining for CD34 was carried out precisely as described elsewhere (Wulff et al., 2001a). For 3β-HSD, endogenous peroxidase activity was quenched with a 30-min incubation in 3% hydrogen peroxide. A normal goat serum (NGS, 1:5 dilution) block was used, and sections were incubated overnight at 4°C in the first antibody. Immunolocalization was undertaken using the mouse EnVision kit (Dako, Glostrup, Denmark). For signal detection, liquid diaminobenzidine substrate (DAB, Dako) was used. Sections were counterstained with haematoxylin.

17α-Hydroxylase immunostaining was carried out using avidin–biotin complex (ABC) amplification. After dewaxing and rehydration, endogenous peroxidase activity was quenched with a 30-min incubation in 3% hydrogen peroxide and a normal porcine serum (NPS, 1:5 dilution in TBS) was applied for 30 min at room temperature followed by an avidin–biotin block (Avidin/Biotin blocking kit, Vector Laboratories, Burlingame, CA, USA; Vector SP-2001) for 15 min. Polyclonal rabbit anti-17α-hydroxylase antibody was applied in NPS at 4°C overnight. As a secondary antibody, swine anti-rabbit biotinylated antibody was used in a 1:500 dilution in TBS for 30 min at room temperature followed by preconjugated horse-radish peroxidase avidin–biotin complex (ABC–HRP, Dako) in 0.05 mol/l Tris, pH 7.4 for 30 min at room temperature. DAB substrate was used to visualize immunostaining, and sections were counterstained in haematoxylin.

Staining for occludin and claudins 1 and 5 was obtained using the ABC method with tyramide enhancement. Endogenous peroxidase activity was quenched with a 30-min incubation in 3% hydrogen peroxide. To reduce non-specific staining, we incubated sections for 30 min in normal rabbit serum (NRS, 1:5 dilution) in TBS containing avidin, washed in TBS and incubated for another 30 min in NRS biotin (Avidin/Biotin blocking kit). The slides were incubated overnight at 4°C with the first antibody. After three washes in TBS, the incubation with the secondary antibody (swine anti-rabbit biotinylated antibody, 1:500 dilution, Dako) for 30 min at room temperature followed. For signal amplification, ABC–HRP (Dako) was applied for 30 min at room temperature. Signal enhancement was achieved by exposing the slides to biotinylated tyramide (GenPoint Kit, Dako) for 20 min at room temperature. Tyramide enhancement followed by
ABC–HRP was repeated twice. After the last tyramide incubation, ABC alkaline phosphatase was used before final signal detection with nitro blue tetrazolium (NBT) substrate (Boehringer-Mannheim, Mannheim, Germany). Visualization was performed using 100 μl slide NBT solution containing 45 μl of NBT substrate, 10 ml of NBT buffer, 35 μl of 5-bromo-4-chloro-3-indolyl phosphate and 10 μl of levamisole.

For VE-cadherin staining, the first antibody was applied for 15 min at room temperature in 2% NRS. Signal amplification was carried out using the CSA-System Peroxidase (Dako, K1500). The instructions of the manufacturer were followed precisely.

**Dual staining (immunofluorescence)**

Because occludin and claudin 1 are not endothelial cell-specific proteins, dual staining with the established cell marker CD34 was carried out to identify co-localization to endothelial cells. After antigen retrieval as described above, the first primary antibody to occludin or claudin 1 was applied overnight at 4°C. Slides were washed three times in TBS before incubation with the secondary antibody (fluorescein isothiocyanate-labelled goat anti-rabbit AB, Zymed, 81–6111) used in 1:100 dilution in NGS/TBS for 30 min. After three washes in TBS, the antibody against CD34 was applied overnight at 4°C before incubation for 30 min with the secondary tetramethylrhodamine isothiocyanate-labelled goat anti-mouse antibody (Zymed, 81–6514) diluted 1:100. Slides were mounted with vectashield (Vector Laboratories, Burlington, CA, USA).

**Image analyses**

Quantitative analysis of immunostaining was performed using an image analysing system linked to an Olympus camera, and the data were processed using Image-Pro Plus Version 3.0 for Windows computer program. Two sections of each CL were analysed for each protein (occludin, claudins 1 and 5, VE-cadherin and CD34). The area of expression of these antigens was measured in 10 randomly chosen fields per section using the ×20 lens for each CL by an operator blinded to the stage of the tissue. The area was expressed as a mean value of the number of fields assessed. For the endothelial-specific proteins, claudin 5 and VE-cadherin, the measurements were adjusted in relation to the varying luteal cell size throughout the cycle and after HCG treatment by estimation of the ratio of total CD34 staining to staining for the antigen of interest in each field (Wulff et al., 2001a). The mean of all fields, i.e. 20 fields per CL, was taken as representative for each CL.

For occludin dual staining with CD34, analysis was carried out on 10 randomly chosen fields per tissue section at ×20 magnification. Because of the small size of the endothelial cells, it was impossible to localize staining for CD34 and occludin to the plasma membrane; thus, the visualization of single endothelial cells was difficult. The quantification of staining proved to be optimal by using a grid superimposed on the area of interest. The size of the boxes was chosen (5 μm in length) so that an endothelial cell would just be covered by it. The grid contained 1452 crossing points. CD34 single-stained cells and dual-stained cells were counted as soon as they hit a crossing point. The percentage of occludin-positive endothelial cells was calculated for the total number of counted endothelial hits. The mean of all fields was taken as representative for each CL.

**Statistics**

Data from each method of quantification were statistically analysed. Separate one-way analysis of variance tests were carried out for each set of data, and a Duncan’s multiple range post hoc test was performed. P < 0.05 was taken as the level of significance for each test. Tests were performed using the Statistical Package for the Social Sciences for Windows Version 6.0.

**Results**

During the normal cycle (Figure 2a), occludin was localized continuously to the plasma membrane of granulosa lutein cells (inset) and endothelial cells of granulosa and theca capillaries (Figure 2b) but was not found in theca lutein cells. After HCG treatment (Figure 2c), a reduction of the overall staining for occludin was detected, and this was significant as compared with mid-luteal phase (0.01 ± 0.002 versus 1.3 ± 0.4%) (P < 0.05) (Figure 2d). In mid-luteal phase, the quantitative analyses of dual staining with CD34 revealed that 65 ± 4% of all endothelial cells are positive for occludin staining, whereas after HCG treatment occludin was no longer detectable in the endothelial cell compartment.

Claudin 1 was exclusively localized to the plasma membrane of granulosa lutein cells (Figure 3a). Staining was apparent in parts of the plasma membrane but not in a continuous belt-like formation as observed for occludin (Figure 3a, inset). Dual staining with CD34 (Figure 3b) demonstrated the absence of claudin 1 in the endothelium. During the early luteal phase, the distribution of claudin 1 tended to be less organized compared with the mid- and late luteal phase. After luteal rescue (Figure 3c), claudin 1 was only detectable in the cytoplasm of few granulosa cells. Image analyses revealed a significant reduction (P < 0.05) in the area of claudin 1 staining from 1.38 ± 0.4 in mid-luteal phase to 0.17 ± 0.04% after HCG treatment (Figure 3d).

Claudin 5 was exclusively localized to endothelial cells (Figure 4). In early, mid- and late luteal-phase specimens,
Depicts the irregular claudin 1 protein localization (arrows) in the plasma membrane of granulosa lutein cells (bar = 30 μm). Dual staining (b) of claudin 1 (green fluorescence, arrow) with CD34 (red fluorescence, arrowhead) showed no co-localization. After HCG treatment (c), claudin 1 was suppressed. The quantitative analysis of claudin 1 staining (d) during early luteal (EL), mid-luteal (ML) and late luteal (LL) stages and after HCG treatment confirmed the significant decrease after HCG treatment (*) as compared with all other stages (P < 0.05).

Claudin 5 was mainly detectable in large vessels of the theca endothelium and in granulosa capillaries (Figure 4a). After HCG treatment, claudin 5 expression was reduced especially in the granulosa compartment (Figure 4b). In this compartment, endothelial staining for claudin 5 was significantly reduced (P < 0.05) from 52 ± 5 during mid-luteal phase to 1 ± 0.5% after HCG treatment (Figure 4c), whereas in the theca compartment the change was less pronounced (Figure 4d).

VE-Cadherin was exclusively localized to the capillary endothelium of the granulosa and theca compartment (Figure 5a). After HCG treatment, VE-cadherin protein expression was reduced (inset). Quantitative analyses revealed no significant differences throughout the normal cycle (Figure 5b). However, although during mid-luteal phase 64 ± 7% of the endothelial cells are positive for VE-cadherin, a significant reduction (P < 0.05) of staining was found after HCG treatment, i.e. 10 ± 2% (Figure 5b).

Discussion

Tissue and vascular permeability is regulated by the distribution of intercellular tight junctions and adhesion molecules. We have shown, for the first time, the cellular distribution of VE-cadherin, occludin and claudins 1 and 5 in the human CL. More importantly, we have demonstrated that conditions simulating early pregnancy are associated with marked changes in the distribution of these junctional proteins.

It is clear that the distribution of junctional proteins differs in different cellular compartments within the CL. Claudin 1 was exclusively localized to the granulosa lutein cells and claudin 5 and VE-cadherin to endothelial cells, whereas occludin was present in both granulosa lutein cells and endothelial cells. The specific localization of VE-cadherin and claudin 5 is not surprising. These proteins are localized primarily to the endothelial cells of the microvasculature in other human tissues investigated (Morita et al., 2003; Virgintino et al., 2004; Vorbrodt and Dobrogowska, 2004). In addition, the localization of occludin to both epithelial and endothelial cells is also seen in other tissues (Langbein et al., 2002; Leach et al., 2002). We have localized claudin 1 to the membrane region of granulosa lutein cells, and this distribution is similar to that seen in cells of the human ovarian surface epithelium (Zhu et al., 2004). However, it is not localized to luteal endothelial cells, in contrast to the endothelial cells of the brain and salivary glands (Fujibe et al., 2004; Peppi and Ghabriel, 2004). The reason for this difference is not clear, but overall our study results highlight that in different cellular compartments, a special subset of different adhesion and junctional molecules is recruited for the mediation of cell adhesion and communication.

Little is known about the distribution and regulation of different adhesion and tight junction proteins in the CL in any species. Our observations indicated that during the mid-luteal phase, the tight junction protein claudin 1 was more organized and evenly distributed in the granulosa lutein cell membrane when compared with the early luteal phase. This suggests that when the CL reaches its fully functional state, in the mid-luteal phase, tight junction distribution and organization are complete. Changes to the organization of tight junctions during the remodelling associated with the formation of the fully functional CL suggest that these proteins may be involved in normal luteal development. This is supported by earlier studies in the baboon CL in which the tight junction molecule ZO-1 was localized to lutein cells with an increase in immunoreactivity during mid-luteal phase (Khan-Dawood et al., 1996b). Although it is likely that the junctional proteins are involved in luteal growth and development, we did not detect any quantitative differences in any of the investigated proteins during the normal luteal phase.

This, however, was not the case in the rescued CL of simulated early pregnancy. Claudin 1 and occludin were significantly down-regulated in the granulosa lutein compartment after exposure to HCG in vivo. In addition, luteal rescue by HCG was accompanied by the down-regulation of the adhesion molecules occludin, claudin 5 and VE-cadherin in the endothelial cell compartment. In the rat ovary, an ovulatory dose of HCG has been shown to reduce the junctional proteins E-cadherin and α-catenin (Sundfeldt et al., 2000). It is clear that HCG causes marked decreases in the immunodetectable junctional proteins investigated, in different luteal cellular compartments, during maternal recognition of pregnancy.

It is likely that the change of distribution of intercellular adhesion and tight junction proteins is of particular importance in the function of the CL of early pregnancy. Unfortunately, we are only able to study a short window during the development of the rescued CL so are not able to determine how quickly these changes take place and how long they are maintained for. The reduced junctional proteins in the granulosa lutein cell compartment may be of functional importance to facilitate the release of key steroidogenic cell molecules such as progesterone...
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However, there may be a structural reason for these observations. CL development during luteal rescue is associated with remodelling of different cellular compartments. The granulosa lutein cells enlarge in size and the luteal vasculature increases in volume so that virtually every lutein cell is in contact at least with one capillary (Wulff et al., 2001a). For initiating such tissue remodelling, the loss of cell junctions may be required as an initial step to loosen the intercellular space ensuring invasion and expansion of capillaries within the granulosa lutein cell compartment. Whatever the reason, the same phenomenon is also seen in the vascular compartment.

Figure 4. Immunohistochemical localization of claudin 5 in the corpus luteum (CL). During the normal luteal phase (a, bar = 50 μm), claudin 5 was found in theca and granulosa capillaries. After HCG treatment (b), a reduction of claudin 5 especially in the granulosa compartment was evident. This observation was confirmed by the quantitative analyses of endothelial staining for claudin 5 in the granulosa (c) and theca (d) compartment during end luteal (EL), mid-luteal (ML) and late luteal (LL) stages and after HCG. The * indicates the significant decrease after HCG treatment as compared with all other stages (P < 0.05).

Figure 5. Immunohistochemical localization of vascular endothelial cadherin (VE-cadherin) in the capillaries of the corpus luteum (CL) of normal luteal phase (a, bar = 50 μm). The inset depicts the reduction of VE-cadherin staining after HCG treatment. This is confirmed by the quantitative analysis of endothelial staining for VE-cadherin (b) during end luteal (EL), mid-luteal (ML) and late luteal (LL) stages and after HCG. The * indicates the significant decrease after HCG treatment as compared with all other stages (P < 0.05).
It is very likely that vascular permeability is increased during early pregnancy. This stage of increased permeability may be required to distribute the high amounts of hormones needed to ensure the maintenance of the early pregnancy. Indeed, high doses of HCG significantly increased vascular permeability (Albert et al., 2002; Kitajima et al., 2006), and in rats, this was accompanied by a significant reduction of claudin 5 protein and mRNA expression (Kitajima et al., 2006). It may well be that the reduction of intercellular adhesion by HCG not only allows vascular expansion but also increases vascular permeability. The role of HCG in influencing vascular permeability is highlighted by ovarian hyperstimulation syndrome (OHSS) which is an iatrogenic complication during assisted conception (Budev et al., 2005). It is characterized by a marked increase in capillary permeability leading to third-space fluid sequestration that contributes mostly to the morbidity (such as oedema, ascites, pleural effusion, renal failure and hypotension) of the patients. The syndrome almost always takes place either 3–7 days after exogenous HCG administration to induce ovulation (early onset) or during early pregnancy, 12–17 days after ovulation induction when endogenous HCG rises (late onset) (Orvieto, 2005). Two main strategies are used to reduce the incidence of OHSS—the first is cycle cancellation to avoid the administration of HCG and the second is to freeze rather than replace embryos in women at risk of OHSS (Orvieto, 2005). The increased vascular permeability of OHSS is critically dependent on the action of HCG.

At present, it is unclear whether HCG has a direct or indirect effect on junctional protein expression. As granulosa lutein cells are the primary site of LH/HCG receptors in the human CL (Duncan et al., 1996), it is likely that HCG directly affects occludin and claudin 1 expression in these cells. It is much less likely that the effect of HCG on endothelial cells is a direct effect. Although the presence of LH/HCG receptors in endothelial cells has been described (Lei et al., 1992; Rao et al., 1993), with a possible role in hormone transcytosis through the endothelium (Misrahi et al., 1996), it is unlikely that there are functionally important receptors in the luteal microvasculature (Duncan et al., 1996). What is more important is the regulation of local factors involved in endothelial cell function such as VEGF (Wulff et al., 2001b). Indeed, where HCG was shown to have a direct effect on lung microvascular endothelial cells, the associated increase in vascular permeability appears to be secondary to locally released VEGF (Albert et al., 2002).

It is well known that VEGF is of fundamental importance to the development and regulation of luteal vascularization (Fraser and Duncan, 2005). The inhibition of VEGF prevents the vascularization of the CL initially but can rapidly inhibit progesterone secretion in the presence of a fully developed vascular network (Fraser et al., 2005b). VEGF also stimulates marked increases in vascular permeability (Dvorak, 2002). Indeed, it has been shown that it can cause a loss of tight junction proteins from endothelial cells by stimulating their phosphorylation (Gonzalez-Mariscal et al., 2003). VEGF is stimulated during luteal rescue (Wulff et al., 2000), and higher concentrations of VEGF are seen in OHSS, suggesting that VEGF is of key importance to its pathophysiology (Pellicer et al., 1999; Albert et al., 2002). Furthermore, follicular fluid from patients suffering from severe OHSS induced the destruction of endothelial ZO-1 tight junction protein, which was prevented by VEGF inhibition (Levin et al., 1998). There is good reason therefore to suspect that the HCG effects on endothelial cells are mediated by locally synthesized permeability factors.

To our knowledge, this is the first report of localization and changes of specific adhesion and tight junction proteins in the human CL after in vivo application of HCG. The observation of decreased occludin, claudins 1 and 5 and VE-cadherin immunostaining after HCG treatment may suggest a hormonal control of cell junctions, which might be associated with tissue remodelling and increased luteal permeability during early pregnancy.

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