In vitro regulation of sheep ovarian surface epithelium (OSE) proliferation by local ovarian factors

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Abstract The ovarian surface epithelium (OSE) forms a lining around the entire ovary and actively participates in the ovulatory cycle. To investigate how specific growth factors and hormones affect OSE proliferation, the present study used sheep as a model to examine the effects of follicular and luteal products on the proliferation of sheep OSE cells in culture, and to analyse the influences of large antral follicles and corpora lutea (CL) on the expression of gonadotrophin receptors (FSHR and LHR) in the OSE. In the present study, follicular fluids from medium and large follicles, and extracts of corpora leutia stimulated the growth of OSE cells. The results of the present study showed that factors in follicular fluid can induce OSE proliferative activity, and this stimulation effect could not be attributed to steroids in follicular fluid since oestrogen and progesterone treatments failed to stimulate OSE cells. The expression of LH and FSH receptors over large follicles (5 mm or larger) were two and four times higher than those over stroma and CL, respectively. In conclusion, OSE proliferation in cycling sheep is associated with underlying developing follicles and CL, mediated by, at least in part, the up-regulation of gonadotrophin receptors, and facilitated by the action of mitogenic glycopeptides and growth factors, but not steroids.

1. Introduction

Normal OSE cells express receptors for several growth factors such as keratinocyte growth factor (KGF), EGF, HGF, IGF, and TGF-β (Berchuck et al., 1991, 1992; Parrott et al., 2000). Additionally, oestrogen, androgen, and progesterone receptors are expressed in normal OSE cells in humans (Karlan et al., 1995) and rats (Adams and Auersperg, 1981). Several studies showed that steroids increase OSE proliferation in vitro, in a dose-dependent manner (Wright et al., 2005), are present in bovine (Parrott et al., 2001) and human OSE cells (Syed et al., 2001). There are indications that LH and FSH have the ability to stimulate OSE cell proliferation in human and ovine OSE cells (Gubbay et al., 2004).

Follicular fluid (FF) is a major constituent of the mature ovarian follicle; it plays an important role in most of the ovarian physiological processes. The ovarian fluid is made up of a different novel secretion e.g. steroids hormones, peptides and glycosaminoglycans (GAGs), and a plasma membrane, especially its...
proteins. The concentration of hormones within the FF changes in correlation to the stage of follicular development. There are several studies determining steroid contents in FF in different species, specifically after the LH surge (Dicleman et al., 1983; Chaffin et al., 1999). According to Baird and Fraser (1975), the level of steroid hormones in FF differs markedly approaching ovulation. A noticeable shift from oestrogen to progesterone production is a major event in the preovulatory phase (Hillier, 1985). Gonadotrophins, FSH and LH are present in FF and their concentrations increase in preovulatory follicles, although remaining below levels in systemic blood.

An *in vitro* model was used to investigate how specific growth factors and hormones affect OSE proliferation. Rodents have been used in several studies for investigating the function and physiological roles of normal OSE (Gaytan et al., 2005; Burdette et al., 2006); however, ruminant models with reproductive cycles relatively similar to the human cycle are preferable. Sheep OSE have been shown to be an adequate model for *in vitro* studies on human OSE (Gubbay et al., 2004) which is why this study uses them as a model.

The aims of the following study are to examine the effects of follicular and luteal products on the proliferation of OSE cells in culture, and to analyse the influences of large antral follicles and corpora lutea on the expression of gonadotrophin receptors by the OSE.

2. Materials and methods

2.1. OSE isolation and cell culture

Sheep ovaries were collected from adult cycling sheep (visible ovarian activity and corpus luteum present, in the age range of 14–18 months) immediately after slaughter at a local abattoir (Edinburgh, Scotland). Ovaries were transported to the laboratory in a sterile thermos containing culture media of M199/MCDB 105 supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma).

OSE cells were obtained by gently scraping the surface of the ovaries (*n* = 20) using the blunt edge of a sterile scalpel. OSE cells were transferred into sterile flasks (*n* = 4), which were pre-coated with foetal calf serum (FCS), and contained culture media of M199/MCDB 105 (1:1) supplemented with 1 mM l-glutamine, 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. OSE cells were examined with a phase-contrast microscope to ensure sufficient flakes of OSE had been obtained. OSE cells were incubated at 37°C in a humidified incubator with an atmosphere of 5% CO₂ in air for up to 28 days with media changed every 7 days according to Hillier et al. (1998). Confluent cells were normally obtained after three to four weeks.

2.2. Cytokeratin immunocytochemistry (ICC)

To confirm the purity of the cultured cells (epithelial origin), a fraction of the cells were seeded in chamber slides (Sigma–Aldrich Ltd.) for ICC to detect cytookeratin (a marker of epithelial cells). Cells were fixed in ice-cold 80% methanol, air-dried then washed in PBS, followed by incubation for 20 min with 10% normal horse serum and further 30 min with cytokeratin antibody (1:50 mouse monoclonal cytokeratin Clone MNF116 Dako Cytomation, Denmark A/S) at room temperature. Following the washing stages, cells were incubated for 30 min with anti-mouse IgG Biotinylated antibody. Finally, cells were incubated with Vectastain Elite ABC kit (Vector Laboratories, Inc., California, USA) for 30 min then washed in PBS. For visualisation, slides were incubated with DAB for 10 min, then counterstained with haematoxylin.

2.3. Proliferation assay

In order to investigate the effect of steroids and growth factors on OSE cell proliferation, confluent cells were recovered using trypsin EDTA (0.05% trypsin and 0.02% EDTA) for 5 min at 37°C. Fresh media was added to the trypsinised cell pellet and a fraction was taken to be counted using a haemocytometer.

OSE cells were seeded into 96 well plates at a density of 5000 cells per well and cultured for 24 h in media containing serum to ensure cell attachment. Cells were incubated for a further 24 h in serum-free media containing 0.01% bovine serum albumin (BSA). The treatments (each treatment was applied to three different wells) were administrated to the cells and incubated for 72 h.

Follicular fluid was aspirated from sheep ovaries (6–8 mm, *n* = 20) and large (10–12 mm, *n* = 15) bovine antral follicles from the ovaries (n = 18) obtained from a local abattoir. A fraction of the fluid was charcoal-extracted to achieve a steroid-free fluid (van Tol and Bevers, 2001). Extracts from mature bovine corpora lutea (CL) (*n* = 7), progesterone (3 ng/ml, 30 ng/ml, and 3000 ng/ml), 17β-oestradiol (3 ng/ml, 30 ng/ml, and 3000 ng/ml), recombinant human insulin-like growth factor 1 (IGF-1) (100 µg/ml; NHPP, Torrance, California), and FCS (final concentration in culture media, 10%) were used as a positive control for the assay. Luteal extracts were obtained by grinding freshly collected bovine corpora lutea. Ground tissue was filtered, centrifuged and sterilized using sterilization filter, and then stored at −20°C. Doses of steroids were representative of preovulatory follicular fluid levels (reaches levels of 1 µg/ml estradiol (E₂) and 17 µg/ml progesterone (P₄) and circulating levels (range 1 ng/ml E₂ and 30 ng/ml P₄) (Lenton et al., 1988).

OSE cell proliferation was measured using the Cell Titer 96® AqueousOne Solution Proliferation Assay (Promega, Southampton, UK) which is based on the conversion of MTS tetrazolium to a coloured formazan product by viable active cells. Following the instructions of the manufacturer, 20 µl of the MTS/PES solution was added to each well, followed by 3 h incubation in a humidified atmosphere. The amount of formazan converted during this period was measured as absorbance at 490 nm in a spectrophotometer. Cell growth in untreated control cells was assigned a value of 1, and relative cell growth in a treated culture was expressed as fold change over the untreated control cells. Data points are group mean values ± SEM from four independent experiments. For each treatment, mean values were calculated from triplicate wells.

2.4. Real-time PCR for expression of LHR and FSHR

To perform real time reverse transcriptase polymerase chain reaction, OSE cells were obtained by scraping the surface of ovaries from cycling sheep (*n* = 20). Cells were collected from different areas of the ovary: areas overlying large antral follicles (≥5 mm; *n* = 17), areas covering mature corpora lutea.
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(n = 12), and areas over the ovarian stroma (i.e., no visible underlying follicles or CL). Six different pools of cells corresponding to each of the three areas (two different pools for each area) were obtained from 20 animals, and RNA was immediately extracted.

The Tri Reagent (Sigma–Aldrich) method was used to extract the total RNA from suspended ovarian surface epithelial cells. Following the manufacturer’s instructions, RNA samples were purified by a phenol–chloroform extraction followed by precipitation with ethanol. The amount of RNA was estimated by spectrophotometer at 260/280 nm. One microgram of each total RNA was reverse-transcribed using Super Script III Reverse Transcriptase (Invitrogen Corp., Carlsbad), and using random primers (Promega, Madison, WI, USA) and a dNTP mix to generate single-strand cDNA for each sample using the thermocycler (Biomefomera Tgradient). Selection of primers (Table 1) for LHR, FSHR, and 18S was performed using LightCycler Probe Design software (Roche Applied Science, Mannheim, Germany). Quantification of mRNA levels for LHR and FSHR was analysed using a RT-PCR cycler (Stratagene Mx3000P). A portion (1/4) of each cDNA was used for quantitative PCR using a master mix (2X SensiMix DNA kit, Quantace Ltd., London, UK) that included SYBR green solution, SensiMix, and MgCl2. PCR settings were in all cases set at 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. All real-time experiments were run in triplicate, and mean values were used for the determination of mRNA levels. The negative controls consisted of RT-negative (RNA template with no reverse transcriptase enzyme) and RT-H2O (water in place of RNA template). The abundance of each target mRNA was determined relative to the housekeeping gene 18S. Changes in the average fold was calculated with Mx3000P real-time PCR system analysis software (Stratagene) using the cycle threshold for each sample relative to a standard curve constructed from RNA extracted from granulosa cells.

2.5. Statistical analysis

The Kolmogorov–Smirnov test and Box plot graphs were used in order to decide if the data was normally distributed. Data for proliferative response of OSE cells to different treatments was presented as mean values from four independent experiments. Results were normally distributed, therefore one-way analysis of variance (ANOVA) was used as a robust test for analysing normally distributed data. After a significant ANOVA, a Tukey post hoc test was performed in order to locate the significant differences between the groups. Analysis was done using Minitab version 15. Data for gene expression was presented as mean values from three different assays, data was not normally distributed in accordance to the Kolmogorov–Smirnov test; therefore, the Kruskal–Wallis test was selected because it is the non-parametric analogue of ANOVA that compares more than two groups. The Dunn’s test was used as a post hoc test to locate the significant differences between the groups. Analysis was conducted using GraphPad Prism statistical software. Differences were considered to be significant at P \leq 0.05.

3. Results

3.1. Cytokeratin expression in OSE cells

To confirm the purity of the epithelial cells in the ovine isolated cells, immunohistochemistry against cytokeratin was performed. Cultured OSE cells stained positively for keratin. Also, the complete intact layer of the OSE was completely stained with the antibody. No negative cells for cytokeratin immunostaining demonstrating the purity of the culture (Fig. 1).

3.2. Proliferative response of cultured OSE cells to follicular fluid and CL extracts

The effects of the main ovarian compartments of follicular fluid and CL on the proliferative activity of the ovine OSE cells were investigated. Cells were grown in the presence of the treatments or medium alone (control). Administration of FCS was used as a positive control for the proliferation assay. Incubation with follicular fluid and CL extracts stimulated the proliferation of cultured OSE an average of about 2.5-fold relative to the control (untreated cells) (P = 0.001). There were no effects of follicle size or previous charcoal extraction in the proliferative response to follicular fluid (P = 0.1) (Fig. 2).

3.3. Effect of steroid hormones and growth factors on the proliferation activity of cultured ovine OSE

Cultured OSE cells were treated with progesterone or oestrogen in a number of concentrations. Steroids administration at different doses (low and high) had no significant effect on cell proliferation (P = 0.1). Only IGF-1 administration induced OSE cells proliferation (mean of 2.2-fold over control; P = 0.01) (Fig. 3).

3.4. FSHR and LHR expression

To investigate whether gene expression of FSHR and LHR were regulated by the underlying ovarian compartments, the levels of FSHR and LHR mRNA in the OSE cells (isolated from different areas around the ovary) were determined. Both receptors (LHR and FSHR) showed a variation in the level of their expression. Distribution of FSHR was statistically varied between the three areas. The highest expression was detected in follicles overlying the ovarian stroma

<table>
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<tr>
<th>Table 1</th>
<th>Primers for real-time PCR.</th>
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<tr>
<td>Gene</td>
<td>Forward primer</td>
</tr>
<tr>
<td>LHR</td>
<td>5'-TCTTTTGCTGAGGAGCTGACTGTA-3'</td>
</tr>
<tr>
<td>FSHR</td>
<td>5'-CACCCCCCTCCTCCCTACG-3'</td>
</tr>
<tr>
<td>18S</td>
<td>5'-GGGGAATCAGGGTTCG-3'</td>
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Fig. 1. Immunostaining demonstrating the purity of the culture. No negative cells for cytokeratin immunostaining demonstrating the purity of the culture.
mRNA of FSHR significantly decreased ($P \leq 0.01$). OSE cells over the CL expressed very low levels of FSHR (Fig. 4). Although the level of LHR expression was not significantly different among the three areas, the distribution of the receptor was following the same trend of the FSHR with the highest level of LHR expression was reported in OSE cells isolated from the area over the large follicles.

4. Discussion

During every cycle, the OSE over the preovulatory follicles is exposed to high concentrations of steroids, growth factors, and inflammatory reagents. There is evidence that OSE cells proliferate after ovulation in order to repair the ruptured area. Preovulatory follicles are the main source of several hormones and growth factors, and these factors are implicated in the tumourgenesis of the OSE cells through mitogenic effects. The current study investigates the effects of steroid hormones produced in a high concentration by the antral follicles on the proliferative activity of the OSE cells. The results show that factors in follicular fluid can induce OSE proliferative activity, and this stimulation effect could be not attributed to steroids in follicular fluid since oestrogen and progesterone treatments failed to stimulate OSE cells. Other factors within the follicular fluid are responsible for the mitogenic effect of this fluid.

The addition of FCS in the serum-free medium enhanced OSE cell proliferation when compared to controls. FCS seems to contain some undefined compound(s) that stimulates proliferation. Serum supplementation provided essential nutrients (amino acids, vitamins, lipids) and adherence material for growing cells (Masters, 2000). Reports suggest that some undefined serum factors may influence epithelial-mesenchymal cell transformation (Auersperg et al., 2001). Fetuin, a glycoprotein component of FCS that contributes to attachment of the cultured cells, may cause adherence of the epithelial cells in a way that happens in re-epithelialisation of the OSE layer. It has been found that Fetuin-A, a Ca$^{2+}$-dependent adhesion factor in FCS induces cell-to-cell adhesion leading to tumorigenesis (Kundranda et al., 2005). Initial adherence
may influence synthesis of cellular Ca\(^{2+}\)-dependent adhesion factors, such as N- and E-cadherins, and these in turn may influence proliferative activity.

In this study, follicular fluids from medium and large follicles and extracts of CL stimulated the growth of OSE cells. Follicular fluid is partially an exudate of serum, and therefore it is expected to contain at least some of the mitogenic factors found in serum.

These extracts or fluids contained a number of anterior pituitary glycopeptide hormones, FSH and LH/hIGF, steroid hormones, oestrogen and progesterone, and several localised growth factors, such as IGF and HGF. In addition, receptors of these follicular modulating hormones are present on the surface of OSE cells. While most of the steroid hormones regulate follicular cycle and maintain luteal cycle, their role in the OSE is not clearly understood. Oestrogens are implicated in OSE neoplastic transformation. In order to examine whether steroidoal hormones affect OSE proliferation, the follicular fluids and CL extracts were processed by passing them through charcoal to remove steroidal components. However, even these steroid-free fluids accelerated OSE proliferation over the serum-free controls. Apparently, steroids have no direct effect on OSE cell multiplication. Steroids may regulate the growth of OSE cells via indirect effects. It has been demonstrated that oestrogen administration induces ovarian cancer cell proliferation by increasing the expression of TGF-\( \alpha \) (Simpson et al., 1998). Previous investigation has indicated mitogenic effects of TGF-\( \alpha \) and EGF on bovine OSE cells (Doraiswamy et al., 2000). In order to further verify the ineffectiveness of steroidal hormones, pure oestrogen (oestradiol) and progesterone at graded concentrations were added to the cultured OSE cells. Neither hormone made any significant difference in proliferative activity of OSE cells over the controls, which is consistent with observations of the human and rhesus OSE cells (Ivarsson et al., 2001; Wright et al., 2003). The results of the current study suggest that factors other than steroids may be responsible for in vitro proliferation of OSE. The likely candidates could be gonadotrophins and the local growth factors produced in follicles, which trigger a sequence of reactions leading to proliferative response.

FSH in particular has been implicated in increasing in vivo OSE proliferation (Choi et al., 2007). However, in vitro results were conflicting, and in various species stimulatory, inhibitory, and non-responsive influences of these hormones were noticed. One explanation for such variation is that the differential expression of surface gonadotrophin receptors and follow-up downstream signalling molecules affect proliferation. It has been reported that hCG and oestradiol may regulate OSE proliferation indirectly through an IGF-I pathway (Wimalasena et al., 1993). In addition, a study in bovine OSE revealed that hCG and FSH could increase the levels of mRNA of KGF and HGF (Shoham, 1994). These growth factors utilise multiple signal pathways to suppress OSE apoptosis, and thus may lead to a mitogenic effect resulting in OSE malignancy. It was a matter of interest to examine whether such localised growth factors have a direct influence in the multiplication of ewe OSE cells. It was observed that relative to controls IGF-1 stimulated the proliferative response over two times. It can be presumed that in cycling ewes the oestrogen response to OSE proliferation could be mediated in part by an increase in the transcription of IGF-1 and HGF. Charcoal stripping is a useful method to eliminate steroids, but also may remove cyto-kines and growth factors from serum (Mckeehan et al., 1984). However, in this study we suggest that charcoal stripping did not remove all growth factors from FF and IGF-1 may be the factor responsible for the stimulatory effect of FF on OSE cell proliferation.

FSH and LH may also mediate through their receptor expression (an independent transcriptional activation of such growth factors), and thus the ultimate manifestation of oestradiol and gonadotrophins is the same. One of the underlying signal transduction operating transcriptional controls is the cAMP-dependent protein kinase A (PKA)-mediated phosphorylation (Gubbay et al., 2006). One factor is the cAMP response element binding protein (CREB) and the activating transcriptional factor-1 (ATF-1). In sheep, CREB/ATF-1 accounted for the survival of OSE through the stimulation of proliferative activity, and the prevention of apoptosis. OSE cells are exposed to the gonadotrophin inflammatory response during ovulation, and cytokines e.g. interleukins-1, -6 may induce oncogenic response. Elimination of such cells by apoptosis at the rupture site during post-ovulatory epithelialisation is presumably an oncoprotective mechanism. In the surrounding regions, gonadotrophins stimulate an opposite response by evading apoptosis and up-regulating CREB/ATF-1 expression. Thus collateral OSE cells take over the rupture site and contribute to the healing process. In humans, the gonadotrophin cAMP-PKA signalling pathway was found to down-regulate N-cadherin protein (Pon et al., 2005). N-cadherin, a Ca\(^{2+}\)-dependent cell-to-cell adhesion protein, was found to be controlling the survival capabilities of OSE. In the absence of adhesion due to N-cadherin down-regulation, the cells were non-aggregated and had a tendency to undergo apoptosis. This feature is observed at the postovulatory rupture site. Thus follicular hormones appear to have both antagonistic effects on OSE cells at the rupture site and in the surrounding region.

Although the expression of gonadotrophin receptors in the OSE has been reported in several species (Parrott et al., 2001; Syed et al., 2001), the distribution of receptor expression across the ovarian surface has not previously been considered. The expression of LH and FSH receptors over large follicles (5 mm or larger) were two and four times higher than those over stroma and CL, respectively, suggesting that OSE proliferation over growing follicles results at least in part from a local increase in the sensitivity of OSE cells to circulating gonadotrophins. It has been reported that treatment of anestrus sheep with oestradiol increased LH receptor expression in the OSE (Murdoch et al., 1999), and in granulosa cells both oestradiol and IGF-I up-regulate the expression of gonadotrophin receptors (Knecht et al., 1984; Hirakawa et al., 1999). Therefore, an increase in gonadotrophin receptor expression in the OSE overlying large follicles may be mediated by paracrine follicular influences.

In conclusion, OSE proliferation in cycling sheep is associated with underlying developing follicles and CL, mediated by, at least in part, the up-regulation of gonadotrophin receptors, and facilitated by the action of mitogenic glycopeptides and growth factors, but not steroids.

References


