Regulation of 3β-hydroxysteroid dehydrogenase type 1 and type 2 gene expression and function in the human ovarian surface epithelium by cytokines

Georgia Papacleovoulou, Kirsten Hogg, K. Scott Fegan, Hilary O.D. Critchley, Stephen G. Hillier, and J. Ian Mason

Centre for Reproductive Biology, Reproductive and Developmental Sciences, The Queen’s Medical Research Institute, University of Edinburgh, 47 Little France Crescent, Edinburgh EH16 4TJ, UK

1Correspondence address. Tel: +44-131-242-6442; Fax: +44-131-242-6441; E-mail: j.i.mason@ed.ac.uk

ABSTRACT: The human ovarian surface epithelium (hOSE) is a squamous-to-cuboidal layer that surrounds the ovary. hOSE undergoes injury and repair cycles as a result of ovulation-induced inflammation, an event relevant to the development of epithelial ovarian cancer (EOC). Locally produced steroids mediate the response to inflammation. 3β-Hydroxysteroid dehydrogenase (3β-HSD) drives the intracellular generation of progestogens and androgens that potentially affect cell survival and proliferation. We therefore investigated the regulation of 3β-HSD along with downstream steroid signalling in hOSE. Double immunofluorescence of cultured primary hOSE cells confirmed the expression of 3β-HSD protein Interleukin (IL). IL-1α treatment of primary cells to mimic ovulation-associated inflammation suppressed 3β-HSD1 expression and stimulated 3β-HSD2 mRNA (P < 0.001), without affecting total 3β-HSD protein and activity or androgen or progesterone receptor (PR) mRNA levels. Conversely, IL-4 as a proxy for a post-ovulatory healing cytokine increased both 3β-HSD transcripts, total protein and activity (P < 0.01). IL-4 also suppressed androgen receptor expression (P < 0.01) without affecting that of the PR, thereby potentially sustaining both progesterone biosynthesis and its underlying signalling in the ovarian surface. 3β-HSD protein was immunodetectable in primary ascites of women who were diagnosed with EOC but both mRNA transcripts were diminished relative to normal cells (P < 0.05). Notably, this difference was countered by IL-4 treatment (P < 0.01). We conclude that stimulation by IL-4 could be physiologically relevant to post-ovulatory ovarian healing and suggest a novel therapeutic strategy for the activation of progesterone-associated apoptosis in ovarian cancer. Also, our results suggest an attenuation of 3β-HSD expression in EOC although further studies are required for confirmation.

Key words: human ovarian surface epithelium / 3β-HSD / ovulation / cytokines / ovarian cancer

Introduction

The human ovarian surface epithelium (hOSE) is a squamous-to-cuboidal cellular monolayer of mesodermal origin that lines the ovary (Gillett et al., 1991). Interest in hOSE arises from the widely held belief that >85% of ovarian cancers are epithelial ovarian cancers (EOCs) (Auersperg et al., 1991; Runnebaum and Stickeler 2001). At least three interrelated but not mutually exclusive hypotheses have been advanced to explain the development of EOC. Natural reproductive events such as ovulation, accompanied by inflammation and fuelled by pre- and post-ovulatory reproductive hormones (i.e. gonadotrophins, estrogens, androgens, progesterone) are believed to have a role in the aetiology of EOC (Fathalla, 1971; Cramer and Welch, 1983; Espey, 1994; Risch, 1998; Ness and Coutreau 1999). Ovulation resembles a controlled acute inflammatory reaction (Espey, 1980), involving inflammatory mediators and proteases necessary for follicular rupture and post-ovulatory remodelling (Bonello et al., 1996; de Visser and Coussens, 2005). Proteolytic breakdown of the follicle wall and inflammatory necrosis of adjacent surface epithelial cells are followed by re-epithelization and associated susceptibility to DNA damage (Murdoch et al., 1997). Thus, each ovulatory episode potentially leads to the accumulation of harmful genetic mutations and subsequent neoplastic transformation of hOSE (Ness et al., 2000a, b; Murdoch et al., 2001). Elucidating the local anti-inflammatory steroidial mechanisms that normally protect OSE at ovulation could therefore potentially reveal novel molecular markers for diagnosing or treating ovarian cancer (Rae and Hillier, 2005).

We previously reported that the OSE expresses notable levels of 3β-hydroxysteroid dehydrogenase (3β-HSD) protein with 3β-HSD1 mRNA predominating over 3β-HSD2 mRNA (Papacleovoulou et al., 1999).
inflammation is unknown, but total 3β-HSD activity is also present in ovarian cancers, relative 3β-HSD2 mRNA levels are lower than in normal hOSE cells, suggestive of an acquired feature of neoplastic transformation.

**Methods**

**Subjects**

The physiology of the ovarian cell surface was studied using primary hOSE cells and ovarian tissue that were obtained from premenopausal women (mean age 36 years) who underwent surgery for benign gynaecological disorders, including fibroids, heavy menstruation and pelvic pain. Subjects with endometriosis or receiving any medication, including oral contraceptives, that could potentially affect ovarian function were excluded. Ovarian surface epithelial cells were ‘brushed’ from the ovarian surface at an early stage of the surgery to minimize any contamination with blood and stromal cells. The basic clinical profile of these patients is given in Table I. Ovarian cancer cells for in vitro experimentation as well as for histological studies were recruited from post-menopausal and two premenopausal women diagnosed with ovarian cancer (mean age 63 years). Basic clinical information of the ovarian cancer patients is given in Table II. Formal written consent was obtained from all subjects and the project was approved by the Lothian Research Ethical Committee (project numbers 04/S1103/36 and 05/S1103/14 for hOSE cells and 04/S1103/44 for cancer cells).

**Cell lines**

OSE-C2 and hTERT OSE cell lines have been established by immortalization of hOSE cells collected from premenopausal women undergoing...
surgery for non-malignant gynaecological disorders (Davies et al., 2003; Alvero et al., 2004) and were kindly provided by Dr Richard J. Edmondson, University of Newcastle upon Tyne and Dr Eva Sapi, Yale University, CT, USA, respectively. The EOC cell lines, SKOV-3 and PEO14, arise from the ascitic fluid of patients who were diagnosed with EOC. SKOV-3 cell line was purchased from European Collection of Cell Cultures (ECACC, Porton Down, Salisbury, Wiltshire, UK), whereas the PEO14 cell line was a kind gift of Professor Pujol, INSERM, Mont-Pellier, France.

Establishment of primary cell monolayers

Primary hOSE cells and primary cancer cells were collected and cultured as described previously (Kruk et al., 1990; Hillier et al., 1998; Dunfield et al., 2002). Briefly, normal epithelial cell scrapings and ascitic fluid samples were placed into 75 cm² donor-calf serum pre-coated flasks (Corning Inc. Glass Works, Corning, NY, USA) containing MCDB 105/M199 culture medium (1:1 v/v), 15% (v/v) fetal bovine serum (FBS), 2 mmol/l glutamine, 50 μg/ml streptomycin and 50 IU/ml penicillin (all from Life Technologies, Inc., Paisley, UK and Sigma Chemical Co., Poole, UK). Incubations were in a humidified tissue culture incubator gassed with 95% air–5% CO₂ at 37°C. Medium was refreshed every 7 days and when confluent, cells were passaged as described previously (Davies et al., 2003; Papacleovoulou et al., 2009). Culture medium was changed every 3 days and when confluent, cells were passaged as described earlier. Cell viability ranged from 95 to 98%.

**Establishment of cell-line monolayers**

Cell lines were cultured as primary cells with minor modifications. Cells were grown in T162 (Corning) instead of T75 plastic flasks in antibiotic-free culture medium that contained 10% instead of 15% FBS. All cell lines were grown in a 95% O₂/5% CO₂ incubator at 37°C with the exception of OSE-C2 cells which were grown at 33°C as described previously (Davies et al., 2003; Papacleovoulou et al., 2009). Culture medium was changed every 3 days and when confluent, cells were passaged as described earlier. Cell viability ranged from 95 to 98%.

**Histological studies**

**Colourmetric immunohistochemistry**

Paraffin-wax-embedded tissues were cut into 3-μm sections and deparaffinized in xylene, serial dilutions of ethanol, PBS (Sigma, Poole, UK) and distilled H₂O. Permeabilization was achieved with microwaving, in 0.01 M sodium citrate buffer pH 6.0 (Sigma) for 15 min followed by cooling for a further 20 min. Sequential blocking steps of endogenous peroxidase [3% H₂O₂ in dH₂O (v/v); Sigma] and avidin, biotin (Vector, Peterborough, UK) were performed. Each step was followed by PBS washes (2 x). Afterwards, slides were submitted to blocking with non-immune normal goat serum (NGS; Sigma) diluted in PBS (1:5 v/v) containing 5% (w/v) bovine serum albumin (BSA; Sigma) for 20 min followed by overnight incubation with a rabbit polyclonal antibody raised against human recombinant 3β-HSD2 (Thomas et al., 2002) diluted 1:4000 or against anti-human mouse monoclonal cytokeratin 7 (Dako, Ely, Cambridgeshire, UK) diluted 1:1000 in NGS/PBS/BSA at 4°C. Negative antibody staining controls incubated with unconjugated rabbit or mouse IgG₁ antibody (Vector, Peterborough, UK) were run routinely at matched concentrations. Sequential

<table>
<thead>
<tr>
<th>IHC</th>
<th>Age</th>
<th>Status</th>
<th>Histology</th>
<th>CA 125 (μ/ml)</th>
<th>Stage</th>
<th>Grade</th>
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<tbody>
<tr>
<td>Ascites 1</td>
<td>88</td>
<td>Post-menopausal</td>
<td>Serous/endothelioid</td>
<td>202</td>
<td>IIIB</td>
<td>2</td>
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<tr>
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<td>Serous</td>
<td>447</td>
<td>IV</td>
<td>3</td>
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<tr>
<td>Ascites 3 (Fig. 6B)</td>
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<td>Post-menopausal</td>
<td>Endothelioid</td>
<td>3101</td>
<td>IV</td>
<td>3</td>
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<tr>
<td>Solid tumour 1</td>
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<td>Post-menopausal</td>
<td>Papillary serous with minor mixed components</td>
<td>21480</td>
<td>IIIC</td>
<td>3</td>
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<td>Premenopausal</td>
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<td>17912</td>
<td>IIIC</td>
<td>3</td>
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<tr>
<td>Solid tumour 3</td>
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<td>Premenopausal</td>
<td>Metastasis from breast</td>
<td>46</td>
<td></td>
<td>3</td>
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<tr>
<td>Solid tumour 4 (Fig. 6A)</td>
<td>59</td>
<td>Post-menopausal</td>
<td>Poorly differentiated serous</td>
<td>1387</td>
<td>IIIC</td>
<td>3</td>
</tr>
</tbody>
</table>

hOSE versus ascites

| Ascites 4 | 55 | Post-menopausal | Papillary serous                        | 2647          | IIIC  | 3     |
| Ascites 5 | 74 | Post-menopausal | Poorly differentiated serous/endothelioid | 305           | IIIC  | 3     |
| Ascites 6 (as tumour 3) | 41 | Premenopausal | Metastasis from breast                   | 46            |       | 3     |
| Ascites 7 | 67 | Post-menopausal | Serous                                | 353           | IIIC  | 3     |
| Ascites 1 | 88 | Post-menopausal | Serous/endothelioid                    | 202           | IIIB  | 2     |
| Ascites 2 | 73 | Post-menopausal | Serous                                | 447           | IV    | 3     |

IL-4 treatment

| Ascites 6 | 41 | Premenopausal | Metastasis from breast                   | 46            |       | 3     |
| Ascites 8 | 53 | Premenopausal | Serous                                | 447           | IV    | 3     |
| Ascites 2 | 73 | Post-menopausal | Serous                                | 270           | IV    | 3     |

*Stage of this cancer not relevant as it comes from a breast high grade carcinoma.
incubations with goat anti-rabbit or goat anti-mouse biotinylated IgG1 (Vector) and RTU-ABC elite kit (avidin–biotin complex; HRP conjugated; Vector) were each done for 1 h before washing away excess antibody with PBS supplemented with 0.05% Tween 20 (v/v) (PBST; Sigma). Sections were washed thrice with PBST between steps. Finally, slides were incubated with HRP-conjugated diamobenzidine (DAB; Vector) chromagen for 5 min, and after haematoxylin counterstaining, dehydrations in serial ethanol dilutions (50–100%) and xylene were conducted. Antral ovarian follicles served as positive controls for 3β-HSD (Suzuki et al., 1993). The immunostained sections were visualized with an Olympus Provis microscope (Olympus Optical, London, UK).

Fluorescent immunohistochemistry

Cells were passaged into eight-well glass chamber slides (Nunc Lab-Tek, Leicester, UK) at a density of 25 000 cells/well and incubated at 37°C/5% CO2 for 24 h. Following serum-starvation overnight with medium containing 0.01% BSA (Sigma) instead of FBS, cells were fixed in 100% ice-cold methanol at −20°C for 10 min followed by permeabilization with ice-cold acetone at −20°C for 1 min. Sections of ovarian tissues and freshly isolated ascites pellets were processed as stated earlier with minor modifications. Incubation with 3β-HSD antibody or unconjugated rabbit IgG antibody (negative control) diluted in 1:400 in NGS/PBS/BSA preceded immunodetection with goat anti-rabbit IgG1 antibody directly conjugated to Alexa Fluor 488 (1:200 in PBS; Molecular Probes, Poort Gebouw, The Netherlands), which provides red fluorescent positive staining. After non-immune blocking, incubation with the anti-human mouse monoclonal cytokeratin 7 antibody at 4°C overnight (1:300 in NGS/PBS/BSA) followed by immunodetection with goat anti-mouse IgG1 antibody conjugated to Alexa Fluor 488 (1:200 in PBS, Molecular Probes) was performed to yield green fluorescent cytokeratin-positive staining. Slides were counterstained with a nuclear-specific blue fluorescent label, 4,6-diamidino-2-phenylindole (DAPI; Sigma) in 1:1000 dilution in PBS. Mounting was achieved with Permount Aqueous Medium (Beckman Coulter, High Wycombe, Buckinghamshire, UK). Images were captured with Zeiss LSM 510 Meta Axiovert 100M confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) at ×40 magnification.

Experimental treatments of hOSE and cancer cells

Cell suspensions were adjusted to 3.5 × 10^5 viable cells per well of 6-well culture plates for mRNA and protein studies or 10^5 viable cells per well of 12-well plates for 3β-HSD activity assay (Corning). Following cell monolayer establishment (24 h), medium was replaced with serum-free medium for 24 h. Cells were exposed to 0.02 or 0.5 ng/ml of IL-1b for 24 h. Cells were passaged into eight-well glass chamber slides (Nunc Lab-Tek, West Sussex, UK) supplemented with 0.01% (v/v) β-mercaptoethanol (Sigma) and vigorous vortexing. Lysates were then processed for RNA purification using the RNeasy Mini kit (Qiagen) as per manufacturer’s guidelines. To preclude genomic contamination, DNase digestion was performed using the Qiagen RNase-free DNase set, on-column, during RNA purification. Purified RNA (1.5 μl) was quantified using a Nanodrop spectrophotometer (ND-1000, Nanodrop Technologies Inc., Wilmington, DE, USA), with purity ascertained by the 260/280 and 260/230 ratios (above 2.0).

Taqman real-time quantitative PCR mRNA analysis

Two-step Taqman quantitative PCR (qPCR) was performed to measure the levels of 3β-HSD1, 3β-HSD2, PR, AR and IL-4 receptor (IL-4R) mRNA in treated and untreated hOSE and cancer cell samples. Using a first-strand cdNA synthesis kit (Applied Biosystems, Applied, Warrington, UK), DNase-treated RNA was reverse-transcribed to cdNA using random hexamer priming system in a 10 μl reaction mixture as per supplier’s guidelines. cdNA (2 μl) was used for qPCR, using commercial Applied Biosystems reagents. Each reaction was carried out in duplicate. Primer/probe sets for 3β-HSD1, 3β-HSD2 and IL-4R mRNA transcripts were pre-validated (Assay-on-Demand, Applied Biosystems), whereas those for AR and PR were designed using the Primer-Express software (Perkin-Elmer, Beaconsfield, Bucks, UK). Nucleotide sequences are illustrated in Table III. 18S ribosomal RNA served as internal control for each individual sample. Mean values reflecting the PCR cycle when the target transcript started to be accumulated relative to 18S (mean ΔCt in a 40 cycle PCR reaction) are given in Table IV. Values of more than 36 out of 40 PCR cycles were assessed as beyond the limit of robust detection; however, they were included in analysis for comparison reasons. Taqman reactions were performed in an ABI Prism 7900 Sequence Detector (Applied Biosystems).

Western immunoblotting

hOSE cell monolayers were washed with cold PBS and lysed in 50 mM Tris–HCl, pH 7.6, 0.1% SDS, 1% deoxycholate (all from Sigma) containing a cocktail of proteinase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). Cell extracts were then prepared by sonication. Total protein (25 μg/μl) was size-fractionated by electrophoresis (12% SDS/PAGE) and transferred to PVDF membrane (Millipore, Bedford, MA, USA) followed by blocking for 2 h in 5% dried semi-skimmed milk diluted in PBST. Overnight incubation at 4°C with anti-human rabbit 3β-HSD (1:20 000) or anti-human rabbit AR (1:1000) or anti-human rabbit actin (1:1000; Abcam, Cambridge, UK) diluted in 1% dried semi-skimmed milk/PBST was followed by 1-h incubation with goat anti-rabbit IgG1 conjugated to HRP (Sigma) in 1:10 000 in 1% milk/PBST. Proteins were detected by the ECL detection kit (Amersham, USA) and rabbit actin by the ECL detection kit (Millipore). Human placenta (Thomas et al., 1988, 1989) and human H295 adrenocortical cells (Raney et al., 1994) were used to ascertain that the 3β-HSD antibody detected both isoforms. The breast cancer T47D cell line was used as positive control for AR (Chalbos et al., 1987; Buchanan et al., 2005).

3β-HSD activity assay

Pre-cultured cell monolayers were prepared for 3β-HSD enzymatic assay as described previously with minor modifications (Gingras et al., 1999). Treatment media were removed and replaced with 1-ml fresh serum-free medium containing 0.5 μM pregnenolone (PREG) and 150 000 cpm/ml [3H]-PREG to monitor pregnenolone to progesterone conversion, reflecting 3β-HSD dehydrogenase/isomerase activity. Blank (no-cell) culture wells were treated similarly to establish negative controls. After 8 h incubation, the medium was transferred to tubes containing dichloromethane (8 ml) for the extraction of steroids into the organic phase. Samples were centrifuged at 1000g for 15 min, the aqueous phase removed and the organic phase concentrated under a flow of nitrogen gas. Following reconcentration at 100 μl dichloromethane, samples were applied onto silica gel-pre-coated sheets (PE, SIL G; Whatman, Maidstone, Kent, UK) and chromatographed using chloroform/ethanol (92:8 v/v) as the solvent system. Radiochromatograms were produced using a Bioscan 200 imaging detector (Lablogic Systems, Sheffield, UK).
Table III  Sequences of Taqman primers and probes

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<th>Gene</th>
<th>FW Primer (5’ – 3’)</th>
<th>RV Primer (5’ – 3’)</th>
<th>Probe (5’ FAM-TAMRA3’)</th>
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<td>Assay-on-Demand</td>
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<td>3β-HSD2</td>
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<td>Assay-on-Demand</td>
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<td>AR</td>
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Table IV  Mean dCT levels of 3β-HSD1 and 3β-HSD2 mRNA in various cell types

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<th>Target gene</th>
<th>Mean dCT</th>
<th>hOSE</th>
<th>Ascites</th>
<th>OSE-C2</th>
<th>hTERT</th>
<th>SKOV-3</th>
<th>PEO14</th>
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<tr>
<td>3β-HSD1 mRNA</td>
<td>16.3</td>
<td>21.6</td>
<td>19</td>
<td>22.2</td>
<td>22.6</td>
<td>22.5</td>
<td></td>
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<tr>
<td>3β-HSD2 mRNA</td>
<td>21.7</td>
<td>24.3</td>
<td>25.5</td>
<td>25.6</td>
<td>25.4</td>
<td>25.5</td>
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</tbody>
</table>

Mean dCT represents the PCR cycle when the target gene accumulation started relative to the internal housekeeping 18S transcript.

Figure 1  Expression of 3β-HSD protein in cultured hOSE cells. Double-immunofluorescence showed that 3β-HSD (red) was expressed in situ in the cytoplasm of hOSE cells and co-localizes with epithelial-specific cytokeratin 7 (green; b–d). The same expression pattern was observed in 4-week-old cultured hOSE cells (f–h). Antral ovarian follicles stained positive for 3β-HSD (white arrow head) (j). Nuclei were counterstained with DAPI (blue; a, e, i). Pictures were captured with a Zeiss LSM 510 meta-confocal microscope in ×40 magnification (20 μm scale bars).
Statistical analysis

Data sets are presented as means and standard errors (+ SEM). Basic statistical analysis was performed using the GraphPad Prism 4.00 software (GraphPad Software Inc., San Diego, CA, USA). Repeated measures ANOVA and Newman–Keuls post hoc testing were run for multiple comparisons, whereas two-tailed paired Student’s t-tests were performed for single comparisons. P < 0.05 was taken as significant. All values given in the results and figure legends reflect statistical difference relative to untreated control cells, unless otherwise specified.

Results

Expression of 3β-HSD protein in hOSE

Dual immunofluorescence staining showed that 3β-HSD staining (red) overlapped with epithelial cytokeratin (green) staining in situ in the hOSE layer of whole-ovarian tissue (Fig. 1b–d). A similar pattern of 3β-HSD immunostaining was observed in 4-week-old cultured hOSE cell monolayers (Fig. 1f–h). Follicular cells were also positive for 3β-HSD with an intense staining in theca interna cells (Fig. 1j–l).

Figure 2  Regulation of 3β-HSD1 and 3β-HSD2 mRNA by IL-1α and IL-4. Cultured hOSE cells obtained from four distinct patients were treated for up to 48 h with 0.5 ng/ml IL-1α (A). Another three hOSE samples were exposed in vitro to 0.5 ng/ml IL-4 (B) as described in Methods. Finally, a further five hOSE samples were treated with IL-1α and IL-4 (0.5 ng/ml) for 48 h (C). Graphs represent the mean ± standard error of data obtained from four (A), three (B) and five (C) separate premenopausal women, respectively. (A) n = 4, a: P < 0.001; b: P < 0.001; (B) n = 3, b: P < 0.01; c: P < 0.001; (C) n = 5, a: P < 0.01; b: P < 0.001.
Regulation of 3β-HSD1 and 3β-HSD2 mRNA by IL-1α and IL-4 in primary hOSE cells

In order to further evaluate our previous observation that ovarian wounding and post-ovulatory repair involved 3β-HSD (Papacleovoulou et al., 2009), we challenged hOSE cells in a time-course manner with IL-1α (0.5 ng/ml) as a proxy for ovulation-associated inflammation or IL-4 (0.5 ng/ml) as an agent of post-ovulatory repair. Exposure of cultured hOSE cells retrieved from four patients to IL-1α (0.5 ng/ml) for up to 48 h resulted in a progressive and slow reduction of 3β-HSD1 mRNA with an average 8-fold suppression at 48 h (Fig. 2A upper panel; b: P < 0.001). Simultaneously, IL-1α increased 3β-HSD2 mRNA at least 3-fold from the 12 h time-point with no significant alterations within time (Fig. 2A lower panel; b: P < 0.001). On the other hand, the same dose of IL-4 on hOSE cells retrieved from three further patients substantially increased both gene products. Time-course studies revealed that the IL-4 effect on both 3β-HSD1 and 3β-HSD2 mRNA levels were maximal by 12 h (Fig. 2B; b: P < 0.01; c: P < 0.001). To further elaborate the differential actions of IL-1α and IL-4, their effects on 3β-HSD1 and 3β-HSD2 mRNA were evaluated in hOSE cells recovered from the same patients. In five out of five patients, IL-1α (0.5 ng/ml, 48 h) suppressed 3β-HSD1 mRNA, whereas IL-4 (0.5 ng/ml, 48 h) massively stimulated 3β-HSD1 mRNA (Fig. 2C left-hand panel). On the other hand, although IL-1α up-regulated 3β-HSD2 mRNA, its effect was minimal compared with that of IL-4 (Fig. 2C right hand panel; n = 5, b: P < 0.01; c: P < 0.001).

Regulation of 3β-HSD1 and 3β-HSD2 protein and 3β-HSD enzymatic activity by IL-1α and IL-4 in primary hOSE cells

Total 3β-HSD protein and enzymatic activity in hOSE cell cultures were unaffected by IL-1α but were increased ~3-fold by IL-4 treatment (Fig. 3; n = 7, b: P < 0.001). Dehydroepiandrosterone (DHEA), a substrate for 3β-HSD, showed similar results to those obtained with pregnenolone (data not shown).

Assessment of AR and PR protein and mRNA levels in untreated and cytokine-treated hOSE cells

The presence of AR and PR in hOSE cells was confirmed by immunohistochemistry (IHC) on whole-ovarian sections (data not shown), confirming previously described studies (Edmondson et al., 2002; Li et al., 2003); however, reports are lacking on whether these receptors are affected during post-ovulatory injury and repair. AR and PR mRNA expression were sustained in culture but differentially affected by treatment with IL-1α and IL4. Thus, whereas IL-1α did not affect AR or PR mRNA levels (Fig. 4 upper and lower panels, respectively), IL-4 dose-dependently suppressed AR mRNA (Fig. 5A upper panel; n = 4, P < 0.01) without affecting PR mRNA at 48 h (data not shown). This response was confirmed in time-dependent studies over 48 h incubation with 0.5 ng/ml IL-4 treatment (Fig. 5A lower panel; n = 3, b: P < 0.05). This effect was further confirmed at the protein level, although abrogation of AR protein was observed only with exposure of hOSE cells to a high-dose of IL-4 (5 ng/ml) (Fig. 5B upper blot).

Expression of 3β-HSD protein and 3β-HSD1 and 3β-HSD2 mRNA in ovarian cancer

The potential involvement of 3β-HSD-associated pre-receptor metabolism in ovarian cancer was assessed in solid ovarian tumours (n = 4) and EOC cells from ascites (n = 3) obtained from individual patients (Fig. 6). IHC in representative solid ovarian tumours (poorly differentiated and mixed differentiated with mucinous, serous and endometroid features; Fig. 6A, upper and lower panels, respectively) revealed constant expression of 3β-HSD protein in epithelial cytokeratin-positive cells (Fig. 6B). In freshly isolated ascites, immunofluorescence evaluation with epithelial cytokeratin 7 (green) and 3β-HSD (red) antibodies was performed in order to investigate whether 3β-HSD was expressed in epithelial cells of malignant origin. The expression of 3β-HSD protein was demonstrated exclusively in cytokeratin-positive cells (Fig. 6B–d) and not in other cell contaminants including active mesothelial or immune cells (Fig. 6E, white arrows). The malignant phenotype of freshly isolated ascites cells was confirmed through haematoxylin and eosin staining where cells were characterized by pleomorphic nuclei and high mitotic count (Fig. 6F). All cancer tissue was dissected and evaluated by a consultant pathologist, and malignancy was confirmed in the respective clinical histopathology reports.
Differential expression of $3\beta$-HSD1 and $3\beta$-HSD2 mRNA in primary hOSE and ascites cells as well as immortalized hOSE and EOC cell lines

Relative expression of $3\beta$-HSD transcripts was quantified by Taqman qPCR in cultured hOSE cells isolated from three separate patients, in ascites cells collected from a further three patients as well as in various cell lines in three independent experiments (Fig. 7). Both immortalized cell lines (OSE-C2 and hTERT OSE), both EOC cell lines (SKOV-3 and PEO14) and also primary ascites cells had significantly lower expression levels of $3\beta$-HSD1 mRNA relative to cultured hOSE cells (Fig. 7 upper panel; $n = 3$, b and c: $P < 0.001$). Moreover, the immortalized OSE-C2 cell line expressed $3\beta$-HSD1 mRNA at higher levels than primary ascites, hTERT OSE and EOC cell lines (Fig. 7 upper panel; $n = 3$, b versus c: $P < 0.01$). On the other hand, $3\beta$-HSD2 mRNA was immeasurable in all the cell lines tested. Only primary ascites cells were positive for $3\beta$-HSD2 mRNA (Fig. 7 lower panel).

However, ascites expressed decreased levels of $3\beta$-HSD2 mRNA relative to cultured hOSE cells, although this was only a trend and not determined to be significant (Fig. 7 lower panel; $n = 3$, $P = 0.06$). Raw dC$_T$ values are presented in Table IV.

Isoform expression pattern of $3\beta$-HSD1 and $3\beta$-HSD2 mRNA in primary hOSE and ascites cells

In all hOSE samples tested, there was variability among different patients; however, $3\beta$-HSD2 mRNA was expressed at lower levels than $3\beta$-HSD1 mRNA in all six cases (Fig. 8A upper panel). On the
other hand, there was variability in the pattern of isoform mRNA expression in EOC samples (Fig. 8 centre panel). The combined data showed an average 15-fold greater level of 3β-HSD1 than 3β-HSD2 mRNA in hOSE cells (Fig. 8A lower panel; n = 6, b: P < 0.05). However, any difference in isoform expression pattern was not observed in primary ascites (Fig. 8A lower panel). Quantitative comparisons also showed that 3β-HSD1 mRNA expression was 42 times higher in normal hOSE than in ovarian cancer cells (Fig. 8A lower graph; n = 6, b: P < 0.05). 3β-HSD2 mRNA levels in cancer cells were also decreased 5-fold relative to hOSE cells, though this difference was not statistically significant (Fig. 8A lower graph; n = 6, P = 0.06). As expected, 3β-HSD2 was undetectable in placental samples, whereas 3β-HSD2 was 70 times higher than 3β-HSD1 in granulosa-lutein cells (Fig. 8B upper and lower panels, respectively).

Regulation of 3β-HSD1 and 3β-HSD2 mRNA by IL-4 in ovarian cancer

Having demonstrated 3β-HSD protein and transcriptional activity in malignant cells, although at lower levels relative to hOSE cells, we examined whether low 3β-HSD1 and 3β-HSD2 mRNA expression...
levels could be increased after the treatment of cultured cells established from cancer ascites samples with IL-4 (0.02 and 0.5 ng/ml) for 48 h. We observed IL-4R mRNA expression in cancer cells at levels similar to those found in normal primary hOSE cells (Fig. 9B).

Also, qPCR studies revealed that IL-4 treatment substantially increased both 3β-HSD1 and 3β-HSD2 mRNA levels on average 96-fold (Fig. 9B upper graph; n = 3, b: P < 0.01) and 77-fold (Fig. 9B lower graph; n = 3, b: P < 0.05; c: P < 0.01), respectively. These data demonstrate that aberrant 3β-HSD functionality in cancer can be restored by treatment with IL-4.

**Discussion**

We demonstrate 3β-HSD gene expression and enzymatic activity in hOSE cells, suggestive of an intracrine role for 3β-HSD activity in determining functional cellular responses mediated by AR and PR. As noted in the Introduction, differential regulation of 3β-HSD1 and 3β-HSD2 mRNA levels in these cells by cytokines was noted in previous studies (Rae et al., 2004b; Papacleovoulou et al., 2009). We now establish that both 3β-HSD isoforms likely contribute to the levels of enzymatic activity detected in normal hOSE. Both 3β-HSD mRNA are stimulated by IL-4, followed by a substantial increase in total 3β-HSD protein and activity. The same treatment suppresses AR expression without affecting that of PR. We also find that ovarian cancer cells have lower levels of overall 3β-HSD gene expression but this can be restored by exposure to IL-4.

An advantage of our studies is that we have accumulated data based on OSE and cancer cell function in several patients and therefore they are potentially reflective of (patho)physiological functions of hOSE in vivo. Moreover, our cell culture system based on a 2- to 4-week exposure of the cells to FBS-containing medium prior to experimentation was likely to neutralize the effect of reproductive hormones that might be encountered as a result of cell collection at different stages of the menstrual cycle (Yong et al., 2002; Rae et al., 2004a). Moreover, this culture system was applied to both primary hOSE and cancer cells, and as such, any contribution of the hormonal environment of the cells at the time of collection should be minimal. This appeared essential, since the use of multiple patients to produce enough replicates for each distinct experiment allowed us to overcome restrictions in cell numbers along with limitations in long-term culture of cells from the same subject. We also reinforced these data by the use of various cell lines established from immortalization of primary hOSE and cancer cells, and as such, any contribution of the hormonal environment of the cells at the time of collection should be minimal. This appeared essential, since the use of multiple patients to produce enough replicates for each distinct experiment allowed us to overcome restrictions in cell numbers along with limitations in long-term culture of cells from the same subject. We also reinforced these data by the use of various cell lines established from immortalization of primary hOSE (OSE-C2 and hTERT OSE cell lines) or from the ascitic fluid of patients who were diagnosed with EOC (SKOV-3 and PEO14).

The finding that 3β-HSD is expressed in hOSE and co-localizes with epithelial-specific cytokeratin markers establishes our previous studies showing that hOSE is capable of producing PR- and/or AR-activating steroid ligands (Papacleovoulou et al., 2009). Both AR and PR are expressed along with 3β-HSD in hOSE cells, which agrees with previous studies showing responsiveness of hOSE to androgens and progesterogens (Edmondson et al., 2002; Li et al., 2003). Co-expression of cytokeratins with 3β-HSD in cultured hOSE monolayers implies that hOSE cells keep their epithelial phenotype and capacity to express 3β-HSD in culture, and this allowed us to investigate in vitro the biological functionality of this enzyme in hOSE cell monolayers. 3β-HSD is involved in the biosynthesis of all classic steroid hormones and is essential for tissue homeostasis. This enzyme converts pregnenolone to the potent PR ligand, progesterone and DHEA to the weak AR ligand, androstenedione. Androstenedione can be subsequently reduced to the more potent AR ligand testosterone by 17β-HSD, which is also present in hOSE cells (Rae et al., 2004b). Therefore, our data support the concept that hOSE possesses the enzyme machinery necessary to actively participate in ovarian steroidogenesis (Rae et al., 2004b).

3β-HSD protein and transcriptional activity were also found in solid ovarian tumours and epithelial cells of malignant origin. This implies that ovarian tumours have the capacity to locally generate active progestogens and androgens. Importantly, in double-immunofluorescence studies on ascites samples, it was shown that only epithelial cytokeratin 7-immunopositive cells expressed 3β-HSD. This suggests that our 3β-HSD data for primary ascitic cell cultures most likely reflect malignant epithelial cells rather than other cellular contaminants in the cultures such as activated mesothelial cells. Bearing in mind this potential limitation, comparisons of ascites with primary hOSE cells from patients with benign gynaecological disorders established that 3β-HSD mRNA expression is substantially reduced in ovarian cancer cells. Importantly, this observation was also confirmed in the immortalized OSE-C2 and hTERT OSE cell lines as well as in
SKOV-3 and PEO14 EOC cell lines. Collectively, these data suggest attenuation of pre-receptor metabolism in cancer cells relative to normal hOSE, which might be an acquired feature of (pre)-neoplastic transformation.

qPCR was used to further elucidate the pattern of 3β-HSD isoform expression in primary hOSE and ascites cells. As described previously, in hOSE, 3β-HSD1 mRNA predominated over 3β-HSD2 mRNA (Papacleovoulou et al., 2009), thereby differing from that previously reported for other ovarian compartments, where 3β-HSD2 is the primary isoform expressed (Rheaume et al., 1991). Preponderance of 3β-HSD1 mRNA in hOSE is notable since 3β-HSD1 has a higher catalytic efficiency than 3β-HSD2 (Rheaume et al., 1991). Given that hOSE has limited capacity to produce de novo steroids from cholesterol, at least in vitro (Rembiszewska and Brynczak 1985; Rae et al., 2004b), 3β-HSD1 predominance is consistent with the expression of this isoform in peripheral non-steroidogenic tissues. Unlike hOSE cells, in primary ascites, there was no difference between 3β-HSD1 and 3β-HSD2 mRNA expression levels, although a trend towards increased 3β-HSD1 was noticed. This was probably a result of variability in isoform expression pattern between different patients and the relative small number of the target group.

Both 3β-HSD isoforms are present and regulated in hOSE cells but their relative functional importance has not been distinguished. Exposure of hOSE to pro-inflammatory IL-1α in a time-course mode progressively suppressed 3β-HSD1 mRNA, whereas IL-1α rapidly stimulated 3β-HSD2 mRNA expression. However, total

**Figure 8** Isoform expression pattern of 3β-HSD1 and 3β-HSD2 mRNA in primary hOSE and ascites cells. (A) Combined data of six separate hOSE samples and six separate ascites cells. Taqman qPCR was applied to measure expression pattern of 3β-HSD1 and 3β-HSD2 mRNA. Graphs represent the mean ± standard error of data obtained from six independent replicates (n = 6, b: P < 0.05). (B) Placenta and lutein-granulosa cells were used as positive controls for 3β-HSD1 and 3β-HSD2 mRNA, respectively. IL-4 regulation of 3β-HSD1 and 3β-HSD2 mRNA expression in primary ascites. Cultured ascites cells were treated with 0.02 or 0.5 ng/ml of IL-4 for 48 h as described in Methods. Graphs represent the mean ± standard error of data obtained from three individual patients. Upper graph: n = 3, b: P < 0.01; lower graph: n = 3, b: P < 0.05; c: P < 0.01.
nuclear steroid receptors, could be a functional mechanism to counteract ovulation-associated inflammation and minimize susceptibility of tissue to genetic damage. Androgens such as mibolerone, a synthetic androgen that specifically couples to AR, have been shown to induce cell proliferation and inhibit cell death (Edmondson et al., 2002), whereas progesterone has been shown to be anti-inflammatory (Rae et al., 2004a), apoptotic (Bu et al., 1997; Murdoch and Van Kirk, 2002) and inhibitory to cell growth (Ivarsson et al., 2001) in hOSE and cancer. Therefore, basal levels of androgen synthesis may be essential for post-ovulatory repair that involves DNA cell proliferation, whereas associated progesterone synthesis might be vital to nullify genetically damaged cells. Intriguingly, IL-1α did not affect AR or PR mRNA expression levels, suggesting a balance not only in steroid biosynthesis as discussed earlier but also in the access of the active ligands to the cognate receptors. Thus, the simultaneous pro-inflammatory and anti-inflammatory response of IL-1α to 3β-HSD isoforms with no affect on total 3β-HSD function coupled with the stability of AR and PR expression in response to this stimulus could be an efficient mechanism of hOSE to counteract post-ovulatory inflammation, and could explain in part why ovulation is a scar-free inflammatory reaction.

The ‘anti-inflammatory’ cytokine IL-4 strongly stimulated 3β-HSD1 mRNA and 3β-HSD2 mRNA as well as total 3β-HSD protein and activity in normal hOSE cells and ovarian cancer cells. IL-4 is a Th-2 cytokine that exerts its effects through coupling to IL-4R. Its multifunctional actions including its role in inflammation have been reviewed (Nelms et al., 1999). Up-regulation of both 3β-HSD mRNA and activity, enhancing progesterone and androgen biosynthesis, might be part of the in vivo post-ovulatory healing mechanisms of the ovarian cell surface. Concomitantly, IL-4 attenuated AR mRNA and protein, although in a sluggish manner, with no effect on PR, suggesting that IL-4-induced 3β-HSD favours progesterone rather than androgen signalling and this might be a functional local anti-inflammatory mechanism, given the anti-inflammatory and apoptotic effects of progesterone noted earlier. Interestingly, hOSE cells do not have the capacity to secrete IL-4 de novo but they do express high levels of IL-4R (Burke et al., 1999). Up-regulation of both 3β-HSD mRNA and activity, enhancing progesterone and androgen biosynthesis, might be part of the in vivo post-ovulatory healing mechanisms of the ovarian cell surface. Concomitantly, IL-4 attenuated AR mRNA and protein, although in a sluggish manner, with no effect on PR, suggesting that IL-4-induced 3β-HSD favours progesterone rather than androgen signalling and this might be a functional local anti-inflammatory mechanism, given the anti-inflammatory and apoptotic effects of progesterone noted earlier. Interestingly, hOSE cells do not have the capacity to secrete IL-4 de novo but they do express high levels of IL-4R (Burke et al., 1996). The main source of IL-4 secretion in the premenopausal ovary is the peripheral blood mononuclear cells that infiltrate the peri-ovulatory follicle and later the corpus luteum. IL-4 is highly up-regulated at the luteal phase of the menstrual cycle when progesterone is also abundant (Hashii et al., 1998). Accordingly, during the progestogenic luteal phase, OSE cells stop proliferating, and genetically scarred cells become committed to death pathways (Murdoch and Van Kirk, 2002). Collectively, IL-4 secretion increases at the peri-ovulatory follicle and the progestogenic corpus luteum post-ovulatory. Therefore, elevated IL-4 acts in the 3β-HSD of hOSE in a paracrine manner, thereby inducing progesterone-associated death pathways to secure proliferation of only genetically integral cells for successful repair of the stigma. It is also interesting that IL-4 levels are increased in the corpus luteum of pregnancy (Hashii et al., 1998), which is consistent with the protective role that pregnancy and multiparity have against ovarian cancer (Adami et al., 1994).

The low levels of 3β-HSD expression in ovarian cancer cells and the striking restoration of 3β-HSD1 and 3β-HSD2 mRNA levels by IL-4 in cells of malignant origin are consistent with an anti-tumorigenic role for this cytokine in ovarian cancer. We note that an anti-tumour role of IL-4 in breast cancer has been also demonstrated (Gingras

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**Figure 9** (A) Relative expression of IL-4R mRNA between hOSE and ascites. Basal transcriptional levels were measured in primary hOSE and ascites cell monolayers. Graphs represent the mean ± standard error of data obtained from six individual patients in each group. (B) IL-4 regulation of 3β-HSD1 and 3β-HSD2 mRNA expression in primary ascites. Cultured ascites cells were treated with 0.02 or 0.5 ng/ml of IL-4 for 48 h as described in Methods. Graphs represent the mean ± standard error of data obtained from three individual patients. Upper graph: n = 3, b: P < 0.01; lower graph: n = 3, b: P < 0.05; c: P < 0.01.
et al., 1999; Nagai and Toi, 2000). IL-4 has consistently been reported to attenuate the expression of TNF-α and IL-1α-related pro-inflammatory effects in the TNF-α transgenic mice (Bessis et al., 1998). Similar to hOSE cells, cancer cells do not secrete IL-4 de novo either but they are positive for IL-4R (Burke et al., 1996). Importantly, we did not observe any differences in IL-4R mRNA expression levels between cancer and hOSE cells, suggesting that the absence of the ligand and not the receptor is associated with tumorigenesis. Consistently, serum IL-4 levels have been shown to be reduced in post-menopausal women (Kumru et al., 2004), a stage of life where EOC is most common. It is highly possible, therefore, that IL-4-increased 3β-HSD in favour of local progesterone signalling in hOSE is lost after the menopause. This could have profound implications for the development of post-menopausal ovarian cancer and deserves further investigation. Moreover, it has been reported that estrogens can increase interferon-γ (Th-1 response), resulting in the lack of an increase in serum IL-4 levels (Th-2 response) (Kumru et al., 2004). It is therefore of interest that a positive correlation exists between estrogens and ovarian cancer (Choi et al., 2001; Mukherjee et al., 2005; Beral, 2007).

In summary, we demonstrate the expression and functionality of 3β-HSDs during wound-healing cycles of hOSE. Pro-inflammatory IL-1α does not affect total 3β-HSD, AR and PR expression, attesting to a balance in the intracellular steroid biosynthesis and access of the active ligands to cognate receptors, and this could contribute to the integrity of the ovarian cell surface after post-ovulatory injury. On the other hand, IL-4 can stimulate 3β-HSD expression levels, potentially triggering steroid production in the ovarian milieu and also can suppress AR expression, thereby sustaining just the apoptotic and anti-inflammatory progesterone action. Moreover, IL-4 can counteract attenuated 3β-HSD expression in ovarian cancer. IL-4-increased 3β-HSD could therefore be a critical determinant of progesterone bioavailability and anti-tumorigenic signalling via PR during ovulatory menstrual cycles, with potential application to the treatment of EOC.

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