The influence of blood cells and PDGF on porcine theca cell function in vitro

E.M. Shores, M.G. Hunter*

Division of Animal Physiology, School of Biological Sciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire LE12 5RD, UK

Received 6 March 2000; received in revised form 15 August 2000; accepted 11 September 2000

Abstract

The role of red and white blood cells in the regulation of porcine theca cell function is poorly understood. Interactions between these cell types and a potential mediator of any interaction, PDGF, were investigated using a serum-free culture system. Theca cells were collected from 6–9 mm antral follicles and plated at 50 × 10^3 viable cells/well. In the first experiment, macrophages were removed and theca cells ± macrophages were cultured with a range of PDGF doses (0.1, 1, and 10 ng/ml) ± IGF-1. In the second experiment, red blood cells were removed with lysing buffer. In both experiments the effect of treatment on steroidogenesis and viable cell number was examined. Macrophage removal decreased oestradiol production but increased androstenedione output irrespective of the presence of IGF-1 (oestradiol ± IGF-1, P < 0.001; androstenedione P = 0.02 without IGF-1, P < 0.001 with IGF-1). PDGF increased oestradiol synthesis by whole and macrophage-free theca cell preparations but only in the presence of IGF-1 (P < 0.001). In contrast, androstenedione production was unaffected by PDGF dose in the presence of IGF-1 (P = 0.67). Without IGF-1, 10 ng/ml PDGF tended to decrease androstenedione levels (P = 0.06). Macrophage removal increased viable cell number at 144 h (P < 0.001 ± IGF-1) as did PDGF (P < 0.001 ± IGF-1). In the absence of IGF-1, there was a PDGF × cell type interaction (P = 0.02). Macrophage-free cultures with 10 ng/ml PDGF had twice as many viable cells as whole preparations with no PDGF. In the second experiment, red blood cell removal did not affect steroidogenesis or the number of viable cells present at 144 h when cells were cultured with IGF-1. The data show that theca cell/macrophage interactions do occur, and influence both steroidogenesis and viable cell number during culture. The macrophage product(s) enhanced oestradiol synthesis but reduced androstenedione production and the number of viable cells. As all these interactions were not mimicked by PDGF, PDGF cannot be the only factor mediating the theca/macrophage interaction. When cultured under optimised conditions the presence of red blood cells was not detrimental to theca cell steroidogenesis or the number of viable cells. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Theca cell; Macrophage; Pig-ovary; PDGF; Culture

* Corresponding author. Tel.: +44-115-951-6306; fax: +44-115-951-6302.
E-mail address: morag.hunter@nottingham.ac.uk (M.G. Hunter).
1. Introduction

Concern has been expressed about the possible influence of blood cell contamination in ovarian cell cultures and in particular the potential confounding effects of leukocytes in granulosa cell cultures have been discussed (Beckmann et al., 1991; Best et al., 1994; Evagelatou et al., 1997). The presence of blood cells in granulosa cell cultures is viewed as undesirable because they would not normally be present in this layer within intact follicles. However, extra-vascular leukocytes are present in the theca layer throughout follicular development (Standaert et al., 1991). Despite this, little attention has been paid to the role that red and white blood cells play in the regulation of theca cell function.

Adashi (1990) suggested that leukocytes modulate ovarian function, probably through the secretion of cytokines such as interleukin-1 (IL-1 Macciò et al., 1993; Norman and Brännström, 1996). Macrophages account for approximately 30% of all leukocytes present in the porcine preovulatory follicle (Standaert et al., 1991) and have been implicated as modulators of ovarian cell function (Mori et al., 1989). Macrophages products include IL-1 (Macciò et al., 1993; Norman and Brännström, 1996), tumour necrosis factor-α (TNF-α Best et al., 1994), platelet-derived growth factor (PDGF Ross et al., 1986), fibroblast growth factor (FGF), and transforming growth factors α and β1 (TGFα and TGFβ1) which modulate ovarian cell growth and development (Adashi, 1990). It is possible therefore, that theca cells and leukocytes interact and this interaction may be mediated by a growth factor.

The glycoprotein PDGF is a major mitogen present in serum (Heath, 1992). In addition to platelets, several other cell types secrete PDGF, e.g. macrophages, endothelial cells, vascular smooth muscle and embryonic cells (Ross et al., 1986). The limited range of cells, mostly of mesenchymal origin, that possess PDGF receptors (Scher et al., 1979; Ross et al., 1986) includes theca cells (Duleba et al., 1999). In addition, and despite an apparent lack of receptors, PDGF acts in synergy with FSH to induce LH receptors on porcine granulosa cells in vitro (Mondschein and Schomberg, 1981; Westermark et al., 1983). The effect of PDGF on porcine theca cells however, is unknown.

Cell culture is widely used to study the effects of growth factors on cells. The development of a current physiological serum-free culture system for porcine theca cells in which their follicular phase phenotype and gonadotrophin responsiveness are maintained throughout the culture period is particularly useful (Shores et al., 2000). Cell density plays a vital role in steroidogenesis and viable cell number of ovarian cells in vitro (Campbell et al., 1996; Picton et al., 1999; Shores et al., 2000). Erythrocyte contamination of theca cell preparations is difficult to avoid because of the blood vessel network within this layer. These blood cells may contribute to the overall cell density of theca cell cultures and the effect of red blood cell contamination is unknown.

It was proposed that theca cells and macrophages interact and that this interaction may be mediated by the growth factor PDGF, which is secreted by macrophages. This hypothesis was tested by the addition of a range of PDGF doses to whole and macrophage deficient theca cell preparations. The proposed influence of red blood cells on theca cell function and/or viable cell number in culture was also investigated.
2. Materials and methods

All chemicals were obtained from Sigma, Poole, Dorset, UK unless otherwise stated.

2.1. Cell collection

Theca cells were collected from large (6–9 mm in diameter) morphologically healthy porcine follicles as described by Shores et al. (2000). Briefly, follicles were cut open and the granulosa cells were scraped away. The follicles were rinsed and scraped again. Theca cell sheets were peeled away from the follicle shell and digested with enzymes (collagenase type II, hyaluronidase type I-S) to disperse the cells. Viable cell number before culture was assessed using trypan blue exclusion. The number of red blood cells present in each cell preparation was counted using a haemocytometer. Theca cells were seeded into 96 well plates (Nunclon, Life Technologies, Paisley, Scotland) at a density of 50 × 10^3 viable theca cells per well. Each well contained DMEM/Ham’s F-12 medium supplemented with 10 ng/ml insulin and 0.01 ng/ml LH (USDA-pLH-B2).

2.2. Removal and identification of macrophages

Macrophage removal was based on the method of Beckmann et al. (1991). Briefly, theca cell preparations were incubated for 1 h at 37°C in a 35 mm Petri dish to allow the macrophages to attach to the bottom of the Petri dish. The medium was removed and the dish was washed twice with fresh incubation medium. This medium was pooled and centrifuged to yield theca cells devoid of macrophages. The theca cells were counted again before use in culture. The May-Grünwald Giemsa staining method was used to identify macrophages and to confirm their removal.

2.3. PDGF doses

Whole preparations of cells (whole) or preparations from which the macrophages (−m) had been removed were cultured for 144 h with a range of PDGF doses (0, 0.1, 1 and 10 ng/ml). In addition, wells received either the optimal dose of 100 ng/ml Long-R3 IGF-1 (synthetic IGF-1 analogue, Gropep Pty. Ltd., Adelaide, Australia; the addition of 100ng/ml Long-R3 IGF-1 had previously been shown to maximise steroidogenic output and viable cell number during culture), or to investigate the effect of sub-optimal conditions, no IGF-1 was added (Shores et al., 2000). The spent medium was collected every 48 h and stored at −20°C until assayed for oestradiol and androstenedione.

2.4. Removal of red blood cells

Erythrocytes were removed using a red blood cell lysing buffer (8.3 g/l ammonium chloride in 0.01 M Tris–HCl buffer). Briefly, theca cells were pelleted and mixed with 100 µl of lysing buffer for 1 min. The lysing buffer was washed off and the theca cells resuspended in 1 ml of incubation medium. Viable cell number was assessed before and after addition
of the lysing buffer. Whole cell preparations and red blood cell deficient preparations were cultured for 144 h. The spent medium was collected every 48 h and stored at −20°C until assayed for oestradiol and androstenedione.

2.5. Hormone measurement

Oestradiol and androstenedione concentrations were measured by radioimmunoassay as described previously (Grant et al., 1989; Thomson et al., 1989). Inter and intra-assay coefficients of variation were 13.4 and 6.3% for \( n = 18 \) oestradiol assays and 10.1 and 4.7% for \( n = 6 \) androstenedione assays. The minimum detectable limits were 1.1 pg/tube for oestradiol and 1.3 pg/tube for androstenedione.

2.6. Statistical analysis

Data were subjected to analysis of variance (ANOVA) using the Genstat for Windows computer package. Hormone production was expressed as production/1000 viable cells/48 h. The number of viable cells at 144 h was obtained using the neutral red dye method (Campbell et al., 1996; Picton et al., 1999; Shores et al., 2000). The data were log \((x + 1)\) transformed to remove heterogeneity of variance prior to ANOVA. Replicate cultures were treated as blocks and treatment effects were considered significant at \( P < 0.05 \). The pooled variance was used to calculate the standard error of the difference (S.E.D.) between two means and when indicated by ANOVA, Bonferroni’s test was used to make comparisons between means.

3. Results

3.1. Cell recovery and viability

Using 6–9 mm porcine follicles the average cell yield was \( 3.1 \pm 0.6 \times 10^5 \) viable theca cells \((n = 9 \) preparations\) per follicle and cell viability at recovery was 91.4 ± 6.9%. The average number of red blood cells present was 17.5 ± 1.0 \( \times 10^5 \) per follicle, resulting in a ratio of approximately one theca cell to six red blood cells.

3.2. Removal and identification of macrophages

Macrophages were identified based on their large size (15–20 \( \mu m \) compared with 10 \( \mu m \) for theca cells) and staining pattern, i.e. a large red eccentrically placed nucleus within abundant pale grey blue cytoplasm (Craigmyle, 1986; Burkitt et al., 1993).

It was estimated that there were approximately 300 theca cells to one macrophage in whole cell preparations, giving an estimated 1000 macrophages per 6–9 mm follicle. This would result in approximately 166 macrophages per well of \( 50 \times 10^3 \) viable theca cells.
Fig. 1. The effect of macrophage removal and PDGF dose on oestradiol production by porcine theca cells after 144 h in defined serum-free culture. Theca cells were cultured before (whole) and after (−m) macrophage removal with a range of PDGF doses. The data were log transformed prior to ANOVA and are expressed as production per 10^3 viable cells. The graphs show the mean of three separate cultures each containing four wells per treatment: (a) no IGF-1; (b) 100 ng/ml IGF-1; PDGF dose 0 ( ); 0.1 ng/ml ( ); 1 ng/ml ( ); 10 ng/ml ( ); macrophage removal $P < 0.001$ with or without IGF-1; PDGF dose $P < 0.001$ with IGF-1, $P = 0.33$ without IGF-1.

3.3. Hormone production following macrophage removal

Macrophage removal influenced oestradiol output irrespective of the addition of IGF-1 ($P < 0.001$, Fig. 1a, b). In all cases, macrophage removal was detrimental to oestradiol production reducing it to as little as 60% of whole preparations. Androstenedione levels were also altered by macrophage removal, regardless of IGF-1 status ($P = 0.02$ without IGF-1, Fig. 2a and $P < 0.001$ with IGF-1, Fig. 2b). In all cases and in contrast to oestradiol, macrophage-free cultures produced significantly more androstenedione than whole ones. At 144 h macrophage removal more than doubled androstenedione output.
Fig. 2. The effect of macrophage removal and PDGF dose on androstenedione production by porcine theca cells after 144 h in defined serum-free culture. Theca cells were cultured before (whole) and after (−m) macrophage removal with a range of PDGF doses. The data were log transformed prior to ANOVA and are expressed as production per $10^3$ viable cells. The graphs show the mean of three separate cultures each containing four wells per treatment: (a) no IGF-1; (b) 100 ng/ml IGF-1; PDGF dose 0 (□); 0.1 ng/ml (■); 1 ng/ml (▲); 10 ng/ml (■); macrophage removal $P = 0.02$ without IGF-1 and $P < 0.001$ with IGF-1; PDGF dose $P = 0.67$ with IGF-1; $P = 0.06$ without IGF-1.

3.4. PDGF dose

After 144 h and in the presence of IGF-1, a PDGF dose of 0.1 ng/ml elevated oestradiol production above control ($P < 0.001$) but all other PDGF doses were similar to control levels (Fig. 1). PDGF dose did not affect oestradiol output by whole and macrophage-free preparations when the cells were cultured without IGF-1 ($P = 0.33$, Fig. 1).

Androstenedione output by whole and macrophage-free preparations was not changed by PDGF dose in the presence of IGF-1 ($P = 0.67$, Fig. 2b). After 144 h in the absence of IGF-1, 10 ng/ml PDGF dose tended to produce less androstenedione than the 1 ng/ml dose ($P = 0.06$, Fig. 2a) in both whole and macrophage-free preparations. There was no
Fig. 3. The effect of macrophage removal and PDGF dose on viable theca cell number after 144 h in defined serum-free culture. Theca cells were cultured before (whole) and after (− m) macrophage removal with a range of PDGF doses. The graphs show the mean of three separate cultures each containing four wells per treatment: (a) no IGF-1; (b) 100 ng/ml IGF-1; PDGF dose 0 (□), 0.1 ng/ml (▲); 1 ng/ml (❖); 10 ng/ml (■); macrophage removal P < 0.001 with or without IGF-1; PDGF dose P < 0.001 with or without IGF-1.

3.5. Number of viable cells present after 144 h

Irrespective of the IGF-1 dose present, macrophage-deficient cultures had on average 20% more viable cells at 144 h than whole preparations (P < 0.001 Fig. 3). Viable cell number after 144 h in culture in whole and macrophage-free preparations was affected by PDGF dose regardless of the addition of IGF-1 (P < 0.001 with or without IGF-1). In the absence of IGF-1, the highest PDGF dose (10 ng/ml) resulted in an average of 5000 more viable cells at 144 h than other doses (Fig. 3a). With the addition of IGF-1, the same
Table 1
Hormone production and viable cell number of porcine theca cells following the removal of red blood cells.a, b

<table>
<thead>
<tr>
<th>(a) Hormone production</th>
<th>No IGF-1</th>
<th>100 ng/ml IGF-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>at 144 h (pg/10^3 viable cells/48 h)</td>
<td>Whole −rbc</td>
<td>S.E.D.</td>
</tr>
<tr>
<td>Log oestradiol</td>
<td>0.43c</td>
<td>0.31d</td>
</tr>
<tr>
<td>Log androstenedione</td>
<td>1.64</td>
<td>1.50</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(b) Viable cell number at 144 h</th>
<th>Whole</th>
<th>−rbc</th>
<th>S.E.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(× 10^5 cells)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No IGF-1</td>
<td>8.9c</td>
<td>11.3d</td>
<td>0.9</td>
</tr>
<tr>
<td>With IGF-1</td>
<td>7.9</td>
<td>7.6</td>
<td>0.6</td>
</tr>
</tbody>
</table>

a Porcine theca cells were seeded at 50 × 10^3 viable cells per well in DMEM/Ham’s F12 with 0.01 ng/ml LH and 10 ng/ml insulin. Cells were cultured with or without 100 ng/ml IGF-1. Hormone production was expressed per 1000 viable cells and log transformed prior to ANOVA.

b Values represent the mean of three separate cultures each containing four wells per treatment. Whole: all cell types present; −rbc: red blood cell removed with lysing buffer prior to culture.

c, d Values with the same superscript are not significantly different (P<0.05).

PDGF dose increased the number of viable cells at 144 h. However, the value was not significantly more than that obtained with 1 ng/ml PDGF (Fig. 3b). An interaction between PDGF dose and the cell types present was observed in the absence of IGF-1 (P = 0.02). Macrophage-free cultures treated with 10 ng/ml PDGF contained almost twice as many viable cells after 144 h as whole cell preparations with no PDGF.

3.6. Removal of red blood cells

Red blood cells were completely removed using the lysing buffer. The removal of red blood cells with lysing buffer decreased the theca cell number by approximately 40% (P = 0.003), but did not affect cell viability at the start of culture (P = 0.19 paired t-test).

The removal of red blood cells reduced oestradiol output by 35% at 144 h in the absence of IGF-1 (P = 0.04, Table 1a) but had no significant effect on oestradiol production in the presence of IGF-1 (P > 0.05, Table 1a). Red blood cell removal did not influence androstenedione production either with or without IGF-1 (P > 0.05 at all points, Table 1a). Red blood cell removal increased viable cell number by 25% at 144 h but only in the absence of IGF-1 (P = 0.02, Table 1b). In the presence of IGF-1 viable cell number was similar in the presence or absence of the red blood cells (P = 0.5, Table 1b).

4. Discussion

The results of this study clearly show that theca cells and macrophages interact to influence thecal steroidogenesis and viable cell number during culture. Removal of macrophages decreased oestradiol output, but enhanced androstenedione output. This raises the pos-
sibility that macrophage removal decreased P450 aromatase enzyme activity which led to accumulation of androgen substrate. However, this cannot be the only explanation as oestradiol levels declined by only 21–39% compared with androstenedione level increases of 214–238%. The absolute amounts of androstenedione and oestradiol produced vary considerably (androstenedione up to 10-fold higher than oestradiol). Therefore, even similar percentage changes in the two steroids would reflect a much larger change in the absolute amount of androstenedione synthesised than oestradiol. This provides further strong support for a theca cell/macrophage interaction. Since only theca cells produce androgen macrophage removal must directly affect theca cell function. The action of the macrophage product(s) is 2-fold. Firstly, it inhibits androstenedione production possibly by decreasing P450$_{17\alpha}$ activity, which could be tested by measuring progesterone output. Accumulation of progesterone would suggest that macrophage removal reduced P450$_{17\alpha}$ activity. Secondly, it enhances oestradiol synthesis suggesting a direct action on P450 aromatase. These data may reflect the influence of a single factor acting at two points in the steroidogenic pathway or two distinct factors each acting at one point.

The altered steroidogenesis observed here cannot be accounted for by the removal of other contaminating cell types such as granulosa cells or stromal cells. The current theca cell preparation method results in negligible granulosa cell contamination (Shores et al., 2000). Histochemical staining for 3β-HSD (data not shown) confirmed that contamination by non-steroidogenic cells, such as stromal cells was also minimal.

Viable cell number after 144 h was increased by 15–20% in macrophage deficient cultures compared to whole preparations. It is possible that some cells counted as theca cells in whole preparations are in fact leukocytes. If this is the case macrophage removal would increase the proportion of true theca cells seeded at the start of culture but by less than 1% based on the proportion of macrophages (one macrophage to 300 theca cells) observed here. This cannot account for the increase in viable cell number and therefore, implicates a macrophage product in reducing the number of viable theca cells present at the end of culture.

Two reports have examined the number of macrophages per follicle and suggested values of 30 macrophages per 9 mm$^2$ (pig Standaert et al., 1991) and 5 per 10 mm$^2$ (chicken Barua et al., 1998). The surface area of the theca layer of a 7–8 mm diameter follicle is approximately 500 mm$^2$ giving 250–1700 macrophages per follicle compared to 1000 per follicle estimated in the current study.

PDGF was hypothesised to mediate macrophage/theca cell interactions. Not only is PDGF secreted by macrophages but is also a key mitogen for mesenchymal cells (Ross et al., 1986) and theca cells possess PDGF receptors (Duleba et al., 1999). It has not been possible to find any reports of precisely how much PDGF macrophages secrete. PDGF has been detected in human follicular fluid at an average level of 0.37 ng/ml (McWilliam et al., 1995). Westermark et al. (1983) reported 30–40 ng/ml PDGF in platelet-poor plasma. If all this PDGF were due to macrophage production it would equate to $3 \times 10^{-4}$ ng/macrophage. In the current study there were an estimated 166 macrophages per well. They would therefore, produce 0.05 ng of PDGF. This is similar to the lowest dose of PDGF used in the study, i.e. 0.1 ng/ml which is equivalent to 0.025 ng/well.

The current experiments were carried out in the presence and absence of an optimal dose of IGF-1 to remove the possibility of the optimum culture system (Shores et al., 2000)
masking any effect of PDGF. Differences in the response of the cells to PDGF were observed with and without IGF-1. After 144 h, oestradiol output was increased by 0.1 ng/ml PDGF but only in the presence of IGF-1. This is consistent with reports by May et al. (1992) who showed synergism between IGF and PDGF using porcine theca cells. PDGF did not affect androstenedione synthesis in the presence of IGF-1. In the absence of IGF-1, PDGF generally stimulated androstenedione production. Since macrophage removal enhanced androstenedione production, a macrophage product must decrease androstenedione output. Therefore, PDGF cannot mediate the theca cell/macrophage interaction in terms of androstenedione production.

PDGF increased DNA synthesis by porcine granulosa cells (Hammond and English, 1987) and was mitogenic to porcine theca cells collected from small (1–4 mm) follicles (May et al., 1992). PDGF also stimulated porcine theca cell proliferation and activated kinase signalling pathways in a culture system containing serum (Taylor, 2000). Recent work using rat theca-interstitial cells showed 30–136% increases in DNA synthesis with PDGF doses of 3–30 ng/ml (Duleba et al., 1999). Lack of IGF-1 was previously found to be detrimental to the number of viable theca cells (Shores et al., 2000). PDGF was therefore, able to compensate to some extent for the lack of IGF-1 by increasing viable cell number when a dose of 10 ng/ml was used. In the presence of IGF-1, PDGF treatment increased viable cell number but doses of 1 and 10 ng/ml were not different. This is in agreement with Duleba et al. (1999) who observed additive effects of PDGF and IGF-1 on rat theca cell DNA synthesis. Macrophage removal increased viable cell number at 144 h and therefore, a macrophage product suppressed viable cell number. PDGF cannot mediate this theca cell/macrophage interaction since PDGF increased viable cell number.

In the current study, macrophage product(s) increased oestradiol production, decreased androstenedione output and reduced viable theca cell number. These interactions could not be mimicked by PDGF alone. Other potential candidates include TNFα, which acts directly on P450Δ5α to decrease androstenedione production (Zachow and Terranova, 1994). TGFβ inhibits ovarian cell growth in several species (pig May et al., 1994; Gangrade and May, 1990; cow Roberts and Skinner, 1991; rat Skinner et al., 1987) and stimulates oestradiol synthesis and inhibits androgen production by porcine theca cells (Caubo et al., 1989). Macrophage produced TGFβ (Adashi, 1990), in addition to that produced by porcine granulosa and theca cells (May et al., 1994), could be an important intraovarian factor and further work using the current physiologically relevant culture system would be valuable.

The presence of red blood cells in theca cell cultures is an inevitable consequence of the blood supply to the theca layer. Viable theca cell number and steroidogenesis were previously shown to be lower at high initial cell density (Shores et al., 2000). Red blood cell removal did not effect thecal steroidogenesis or viable cell number when the cells were cultured under optimal conditions, i.e. in the presence of IGF-1. Oestradiol production was reduced at 144 h and viable cell number increased by red blood cell removal but only in the absence of IGF-1. Androstenedione production was unaffected by red blood cell removal at all time points, either with or without IGF-1. These data suggest that red blood cells do not contribute to the overall cell density effect and their presence does not need to be considered in future theca cell culture experiments, particularly when cells are cultured in the presence of IGF-1.
5. Conclusion

In conclusion, the current data indicate that porcine theca cells interact with macrophages. The interaction was characterised by increased oestradiol synthesis, reduced androstenedione output and reduced viable cell number after 144 h of culture in the presence of macrophages. Since it was not possible to mimic all these interactions with PDGF therefore, PDGF was not the sole factor responsible for all the theca cell/macrophage interactions. The presence of red blood cells was not detrimental to theca cells when cultured under optimised conditions.

Acknowledgements

This work was supported by the BBSRC. Thanks are extended to D.G. Bolt, USDA for the provision of porcine LH and to B. Cook for the androstenedione radioactive label.

References


