Temporal relationship between proliferating and apoptotic hormone-producing and endothelial cells in the equine corpus luteum

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Abstract

The temporal relationship between endothelial cell death, vascular regression and the death of hormone-producing cells in the mare has not been established. To determine the dynamics of cell proliferation and death throughout the luteal phase, corpora lutea were studied at the early, mid- and late luteal phase, and after treatment with cloprostenol in the mid-luteal phase to induce premature luteolysis. Changes in cell proliferation and apoptosis were investigated utilising specific markers (phosphorylated histone-3 and activated caspase-3 respectively). Histone-3 positive cells were most abundant during the early luteal phase, and were mainly present in endothelial cells. Histone-3 activity significantly increased in hormone-producing cells 36 h after cloprostenol treatment. Frequency of activated caspase-3 staining peaked on day 14, and was induced by 36 h after cloprostenol administration in mid-luteal phase. However, cell death occurred simultaneously in the endothelial and hormone-producing cells. These results show that a subset of hormone-producing cells enter the early stages of cell division around luteolysis, while the majority of cells are undergoing cell death. Natural and induced functional and structural luteal regression in the mare can be at least partially attributed to simultaneous apoptosis of endothelial and hormone-producing cells. However, there is no evidence that endothelial cell death is the trigger for naturally occurring luteolysis.


Introduction

The corpus luteum (CL) is involved in regulating and controlling the oestrous cycle of the mare (Ginther 1992a). Luteal tissue is heterogeneous and consists of endothelial cells, steroidogenic large and small luteal cells, as well as fibroblasts, smooth muscle cells and immune cells (Reynolds et al. 1994). The life span and function of the CL is regulated by complex interactions between stimulatory (luteotrophic) and inhibitory (luteolytic) mediators (Bachelot & Binart 2005) and these mechanisms vary among species. In humans and other primates, the lifespan of the CL is absolutely dependent on support from pituitary-derived luteinising hormone (LH) (Devoto et al. 2002). However, in domestic ruminants and horses, although the primary luteotrophic hormone is LH (Ginther 1992b, Watson et al. 1995, Berisha & Schams 2005), the termination of luteal activity is caused by uterine prostaglandin factor (PGF)-2α. At the end of the luteal phase, the release of PGF-2α causes a dramatic decrease in plasma progesterone, which is defined as functional luteolysis and precedes structural regression of the CL (Douglas & Ginther 1972, 1976, Henderson & McNatty 1975). A distinct sequence of events is associated with the demise of the CL, including changes in blood supply (Miyamoto et al. 2005), infiltration of leucocytes (Bukovsky et al. 1995, Gaytan et al. 2002), and death of steroidogenic and endothelial cells by both apoptotic (Juengel et al. 1993, McCormack et al. 1998) and non-apoptotic mechanisms (Fraser et al. 1999, Morales et al. 2000, Gaytan et al. 2002). However, the mechanisms involved in regression of luteal tissue in the mare are not fully understood.

PGF-2α or its analogues are widely used clinically to manipulate the oestrous cycle of the mare (Douglas & Ginther 1972, Vanderwall et al. 2000, Nie et al. 2001). However, the early CL is refractory to PGF-2α, limiting its use until after days 5–6 postovulation (Paccamonti et al. 1991). New methods of inducing luteolysis are under investigation in other species. In particular, the regulation of endothelial cell proliferation and function
may be targeted by inhibition of vascular endothelial growth factor (VEGF) using potent antagonists, such as the receptor-based inhibitor VEGF trap. Single injections have proved to be effective in suppressing luteal function at early and mid-luteal phases in the macaque (Fraser et al. 2005), while immunoneutralisation of the VEGF receptor-2 in pregnant mice resulted in premature loss of function and structural luteolysis (Pauli et al. 2005). Therefore, it seems that by reducing or avoiding neovascularisation in the early luteal tissue, luteolysis could be induced at a very early stage. An equivalent development in the mare could provide a therapy for suppression of luteal function and/or induction of luteolysis as early as the day of ovulation.

Development of such therapies for the mare requires more detailed knowledge of events surrounding luteal regression. For example, if a triggering event in luteolysis in the mare involves regression of the endothelium, leading to death of the hormone-producing cells, it may be possible to induce this event prematurely with a VEGF antagonist. In support of this hypothesis, the apoptotic endothelial cell death has been reported as an early event in luteolysis in the sheep and guinea pig (Azmi & O’Shea 1984, Sawyer et al. 1990). In the CL of the mare, Al-zi’abi et al (2002) reported the presence of apoptotic changes around luteolysis. Since that study, more accurate markers are available to investigate cell proliferation and cell death by apoptosis. In particular, the detection of activated caspase-3 has become a reliable indicator of apoptotic cell death in several tissues, including the CL of various species (Boone & Tsang 1998, Rueda et al. 1999, 2000). Furthermore, Carambula et al (2002) demonstrated that the presence of caspase-3 is a prerequisite for apoptosis to proceed normally during luteal regression in mice. Therefore, the detection of caspase-3 can be used to accurately identify apoptotic cells in the regressing equine CL.

As tissue growth and regression is a regulated balance of cell death and cell proliferation, studies on changes in cell death should also evaluate cell proliferation in the same samples. In a previous study, Ki67 was used as a marker of cell proliferation in the equine CL (Al-zi’abi et al. 2002). In addition to establishing that intense angiogenesis occurred during the early luteal phase, the Ki67 antigen was detected in hormone-producing cells during luteal regression (Al-zi’abi et al. 2003). In order to investigate this further, we have utilised a more recently validated marker, phosphorylated histone-3, which identifies cells only at the initial stages of mitosis (prophase and anaphase; Hendzel et al. 1997), and therefore, is more accurate in detecting dividing cells than Ki67. Thus, the aim of the present study was to use new tools to investigate temporal proliferative and apoptotic changes in tissue collected after natural and induced luteolysis. Specifically, the study set out to determine whether endothelial cell death occurred before apoptotic changes were detected in hormone-producing cells.

Materials and Methods

Animals and tissue collection

Fifteen pony mares aged 4–12 years and between 250 and 450 kg body weight were used in this study. During oestrus, ovarian activity of the mares was examined daily by transrectal ultrasonography. The day on which ovulation was detected was considered to be day 0. After appropriate sedation and analgesia, a colpotomy incision was used to remove the ovary containing the CL (Lawler et al. 1999). This study was performed under the approval of the University of Edinburgh Ethical Review Committee and a project licence obtained under the Home Office Animals (Scientific Procedures) Act 1986.

Corpora lutea were obtained at different stages of the oestrous cycle: days 3–4 (early luteal phase; n = 3), day 10 (mid-luteal phase; n = 4), day 14 (early regression; n = 4) and day 17 (late regression; n = 4). In addition, corpora lutea were collected 12 h (n = 4) and 36 h (n = 3) after administration (i.m.) of the PGF-2α analogue, cloprostenol (Estrumate, 263 µg 500 kg, Schering-Plough Animal Health, Middlesex, UK) on day 10 of the oestrous cycle. After surgical removal, the ovaries were immediately transported to the laboratory on ice and the corpora lutea dissected free of connective tissue.

All the luteal tissue samples were fixed in 10% (v/v) neutral phosphate buffered formalin (pH 7.0) for 24 h at

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Area of 100 hormone-producing cells (µm^2) in corpora lutea (CL) at different stages of the luteal phase and after natural and induced luteolysis in the mare.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage of luteal phase</td>
<td>Number of CLs</td>
</tr>
<tr>
<td>Early</td>
<td>3</td>
</tr>
<tr>
<td>Mid</td>
<td>4</td>
</tr>
<tr>
<td>Early regression</td>
<td>4</td>
</tr>
<tr>
<td>Late regression</td>
<td>4</td>
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<tr>
<td>Mid + 12 h post-cloprostenol</td>
<td>4</td>
</tr>
<tr>
<td>Mid + 36 h post-cloprostenol</td>
<td>3</td>
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</tbody>
</table>

Different superscripts (a–c and x–z) indicate statistical differences (P<0.001. Early, days 3–4 postovulation; Mid, day 10 postovulation; Early regression, day 14 postovulation; Late regression, day 17 postovulation. Conversion factor. Because of the hypertrophy of the hormone-producing cells during mid-luteal phase, the number of cells per microscopic field is reduced. The conversion factor was calculated by dividing the cell area at each stage by the value of the stage with the largest area (mid-luteal). This conversion factor represents the proportional change in size related to the size of the cells in the mid-luteal phase. To compensate for this change, the conversion factor shown in the table was multiplied by the number of cells labelled by histone-3 and caspase-3 immunostaining.
room temperature and then embedded in paraffin wax. Serial sections of 4 μm were mounted on to glass microscope slides coated with poly-L-lysine (Sigma Chemical Co.).

**Immunohistochemistry**

For immunocytochemistry, sections were dewaxed in xylene, rehydrated in descending concentrations of ethanol and placed in water. Antigen retrieval was performed by pressure-cooking (Tefal Clypsa Pressure Cooker, Tefal, Essex, UK) sections in 0.01 M citrate buffer, pH 6, for 5 min at high-pressure setting 2. Sections were left for 20 min in hot buffer and washed in Tris-buffered saline (TBS) (0.05 mol/l Tris and 9 g/l NaCl). To determine the localisation and changes in the number of dying cells, an antibody to activated caspase-3 (Asp175) (New England Biolabs, Hitchen, UK) was used. After pressure-cooking, endogenous peroxidase activity was quenched by 5-min incubation in peroxidase block (EnVision horse radish peroxidase (HRP) kit, Dako, Cambridgeshire, UK) at room temperature, then the slides were washed and blocked with normal goat serum (NGS, diluted 1:5 in TBS containing 5% BSA for 30 min at room temperature). Sections were then incubated overnight at 4 °C with cleaved caspase-3 antibody at a 1:100 dilution in NGS. Slides were then washed in TBS and incubated with labelled polymer-HRP as secondary antibody (EnVision kit) for 30 min. Visualisation was achieved by DAB Substrate (EnVision kit): sections were then counterstained with haematoxylin, dehydrated and mounted in Pertex.

Figure 1 Immunostaining of histone-3 in equine luteal tissue of (a) days 3–4, (b) day 14, (c) day 17 and (d) 36 h of cloprostenol administration. Red arrows indicate immunopositive nuclei of luteal cells and black arrows show immunopositive nuclei of endothelial cells. Scale bar represents 40 μm.
Phosphorylated histone-3 localisation was performed as above using anti-phospho histone H3 antibody (Upstate Ltd, Milton Keynes, UK) diluted 1:6000 in normal porcine serum (1:5 dilution in TBS containing 2.5% BSA). Sections lacking the primary antibody were used as negative controls.

**Quantification**

For both histone-3 and caspase-3 immunostaining, ten fields per section were randomly selected and examined blind at 250 magnification. The numbers of labelled cells were recorded. Each labelled cell was classified as either hormone-producing cell or endothelial cell. It was considered that the use of double immunostaining to confirm the identity of hormone-producing cells would not be feasible due to the rapid decline in expression of steroidogenic enzymes in the equine CL, after PG treatment (Beg et al. 2005); therefore, hormone-producing cells were identified based on their characteristic morphology of copious cytoplasm, their polyhedral shape and spherical nucleus, which usually contained a well-defined nucleolus. Endothelial cells had sparse cytoplasm of elongated or variable shape, and a spindle or round nucleus that occupied most of the cell. Changes in cell size during the luteal phase influence the proportion of cells per unit area of tissue. To adjust for this effect, a conversion factor from the measurements of cell area throughout the cycle was used to quantify immunostained cells as described above (Wulff et al. 2001). Sections were examined at ×40 magnification and images captured (Image Pro Plus, Media Cybernetics, Silver Spring Inc., MD, USA Windows). Hormone-producing luteal cells were identified according to their morphological appearance as described above. Ten fields were randomly chosen in each section and the areas of ten cells were measured in each field. The cell areas of hormone-producing cells of corpora lutea collected on day 3 (early), day 10 (mid), day 14 (early regression), day 17 (late regression), and 12 and 36 h after cloprostenol treatment on day 10 were compared.

**Statistical analysis**

All analyses were carried out using S-PLUS (Insightful, Seattle, WA, USA). Overall differences in cell area at different stages of the luteal cycle in the horses were compared using linear mixed effect models. Which mare the cells came from was entered as a random effect to account for the repeated sampling from the same mare and stage of luteal cycle was entered as a fixed effect (Pinheiro & Bates 2000). Preliminary analysis revealed that a quadratic function was required to describe the changes in cell area from early, through mid to late regression. Post hoc analyses were then carried out to ascertain between which phases any significant changes had occurred. Linear mixed effect models were also used to look at the changes in cells area 12 and 36 h after cloprostenol treatment on day 10. The total number of labelled cells in the ten sections examined was compared (after application of the conversion factor described above) independently between mid-luteal (day 10) and all the other stages using Mann–Whitney tests due to the distribution of the data. All results were considered to be statistically significant when P<0.05.

**Results**

Progesterone concentrations and histological appearance of luteal tissue reported elsewhere (Al-zı’abi et al. 2002) confirmed that the functional luteolysis occurred by day 14 (mean plasma progesterone concentration = 0.67±0.17 ng/ml) and was induced by 12 h after PG administration at day 10 of the oestrous cycle (mean plasma progesterone concentration = 1.0±0.01 ng/ml) in all the mares in the group.

**Cell area**

The cell area of hormone-producing cells underwent significant changes during the luteal phase. Cell areas...
were smallest in the early luteal phase and by the mid-luteal phase the cell area had increased to its maximum size before decreasing progressively during natural regression (Table 1). Cell area size also decreased in a linear way following induced luteolysis, and did so within 12 h of administration (Table 1). These changes in cell size were taken into consideration, when evaluating the number of cells labelled by both markers, phosphorylated histone-3 and activated caspase-3. By multiplying with the conversion factor shown in Table 1, overestimation of the number of labelled cells in those stages with smaller cell sizes (e.g., early luteal phase, late regression) was avoided.

**Histone-3 immunostaining**

Phosphorylated histone-3 immunostaining was localised to the nuclei of endothelial and hormone-producing cells throughout the different CL stages analysed (Fig. 1). The number of labelled endothelial cells (Fig. 1a) was significantly higher at days 3–4 compared with day 10 (Fig. 2a), indicating mitotic activity in the early CL associated with neovascularisation. In all the other stages (days 10, 14 and 17) and 12 and 36 h after cloprostenol administration, the number of labelled endothelial nuclei remained low, although a dense network of capillaries was present among luteal cells at all stages. The number of labelled hormone-producing cells was low in the early luteal phase and labelled cells were rare in mid-luteal phase, and 12 h after cloprostenol. However, during regression on days 14 and 17 and 36 h post-cloprostenol, hormone-producing cells staining for histone-3 were clearly evident (Fig. 1b–d). The incidence of such cells was significantly higher 36 h postcloprostenol when compared with day 10 (Fig. 2b).

![Figure 3 Immunostaining of activated caspase-3 in equine luteal tissue of (a) day 10, (b) day 14, (c) day 17 and (d) 36 h of cloprostenol administration. Red arrows indicate immunopositive luteal cells and black arrows show positive endothelial cells. Scale bar represents 40 μm.](image-url)
markers of cell proliferation and apoptosis. Detection of activated caspase-3 by immunostaining confirms that in the mare, apoptosis occurs in both types of cells: hormone-producing and endothelial cells, during the natural and induced process of luteolysis. Contrary to our expectations, an increase in activated caspase-3-labelled cells was detected in both endothelial and hormone-producing cells at the same time points; day 14 of the natural luteal phase and 36 h after cloprostenol injection. Despite inducing luteolysis with cloprostenol and examining the early time point (12 h), when the earliest structural changes associated with luteolysis are taking place (Al-zi’abi et al. 2002), there was no evidence that the endothelial cell death precedes that of hormone-producing cells. Therefore, the hypothesis of endothelial cells dying by apoptosis before the luteal cells could not be supported.

The onset of endothelial cell death, as indicated by the appearance of activated caspase-3 staining, at day 14 and after cloprostenol-induced luteolysis coincides with the disappearance of VEGF mRNA and protein from the hormone-producing cells of the equine CL (Al-zi’abi et al. 2003). This supports the idea that VEGF acts not only as an endothelial cell proliferation factor, but also as a survival factor for recently formed endothelium, and that its downregulation is associated with endothelial cell death in this species. In contrast to the current results in the mare, studies in our laboratory on the regressing human and marmoset CL have revealed that the localisation of activated caspase-3 to endothelial cells to be a very rare event. In addition, in a recent report on the localisation of activated caspase-3 in the regressing CL of the macaque, the specific localisation to endothelial cells was not mentioned (Peluffo et al. 2005). This suggests that in domestic species, in which structural luteolysis is brought about by endogenous uterine PG, the endothelial cell death plays a major role in the luteolytic cascade. By contrast in primates, where there is no endogenous luteolin, luteolysis is a relatively protracted event involving predominantly non-apoptotic mechanisms (Fraser et al. 1999) or necrosis of other luteal cells (Gaytan et al. 2002), and in which apoptosis of endothelial cells is less apparent.

Although dividing cells have been detected in the regressing CL, their frequency was relatively low (Al-zi’abi et al. 2003), and it was thought to be unlikely that quantitative PCR would be sensitive enough to detect proliferation in the tissue. Immunolocalisation of phosphorylated histone-3 proved an excellent marker of cell proliferation in the equine CL and confirmed that intense angiogenesis occurs in the early luteal phase of the mare as in other species studied to date (Jablonska--Shariff et al. 1993, Christenson & Stouffer 1996, Dickson & Fraser 2000). A surprising finding in our previous study (Al-zi’abi et al. 2003) was that cell proliferation, based upon incorporation of Ki67, also occurs in presumptive hormone-producing cells in the regressing luteal tissue

Discussion

This is the first study to analyse the dynamics of luteal tissue at the time of regression in the mare using sensitive

![Figure 4](image-url)
during the late luteal phase and after PG administration. Since proliferation was detected in the same specimens using a marker that is considered the most stringent indicator of cell proliferation, we provided strong evidence that a subset of hormone-producing cells enter the early stages of cell division, while the majority of cells are undergoing cell death. However, the reason for this phenomenon remains to be determined.

In conclusion, although luteolysis is associated with early deletion of the endothelium in some species (Azmi & O’Shea 1984, Sawyer et al. 1990, Modlich et al. 1996, Gaytan et al. 2002), there is no evidence that endothelial cell death is the trigger for naturally occurring luteolysis in the mare, because we have established that the death of both endothelial and hormone-producing cells is temporally associated. However, this does not exclude the possibility that inducing selective regression of the immature vasculature could trigger functional and structural luteolysis as a result of decreased blood supply.

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