Ubiquitin and apoptosis in the corpus luteum of the marmoset monkey (*Callithrix jacchus*)

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The polypeptide ubiquitin covalently binds to cytoplasmic proteins and marks them for proteolytic degradation. Ubiquitin is upregulated during apoptosis in some systems. Apoptosis increases during luteolysis but it is not known whether ubiquitin is expressed in regressing corpora lutea. Marmoset ovaries were removed on day 10 of the luteal phase from animals that had received either no treatment, treatment with the PGF_{2α} analogue cloprostenol 24 h earlier, or treatment with the GnRH antagonist antarelix for either 24 or 48 h before ovary collection. Ubiquitin was localized on ovarian sections by immunocytochemistry, and oligonucleosome formation characteristic of apoptosis was examined in isolated corpora lutea by electrophoresis of extracted [³²P]DNA. Oligonucleosome formation was low in midluteal corpora lutea on day 10 but increased after induced luteal regression with PGF_{2α} and GnRH antagonist. Nuclear ubiquitin immunoreactivity was found in 1.66 ± 0.66 steroidogenic cells and cytoplasmic staining was found in 0.4 ± 0.3 steroidogenic cells (per $\times 40$ field of view) in midluteal phase corpora lutea on day 10. Luteolytic induction with PGF_{2α} significantly increased the number of cells exhibiting cytoplasmic immunoreactivity to 12.24 ± 1.6 ($P < 0.05$). Ubiquitin immunoreactivity was not observed after GnRH-induced luteal regression. Apoptotic oligonucleosome formation was found after induced luteal regression with both PGF_{2α} and GnRH antagonist, but ubiquitin upregulation only occurred after PGF_{2α}-induced regression. These results indicate that ubiquitin expression is not specific for luteolysis and is not an indicator of luteal apoptosis, but that the polypeptide does play a role in luteal cellular responses to PGF_{2α}.

Introduction

The corpus luteum of the ovary develops from a follicle after ovulation of an oocyte. Corpora lutea primarily produce the steroid hormone progesterone that is essential for the establishment and maintenance of pregnancy. If the ovulated oocyte is not fertilized and pregnancy does not occur, the corpus luteum ceases hormone production in a process known as functional luteal regression. It then undergoes structural luteal regression, characterized by cell death and the loss of luteal tissue.

In a non-fertile cycle the corpus luteum undergoes regression after a fixed species-specific period of time. This suggests that luteal cell death is programmed, and a number of studies have shown that luteal regression is associated with an increase in apoptosis (Orlicky *et al.*, 1992; Juengel *et al.*, 1993; Dharmarajan *et al.*, 1994; Kenny *et al.*, 1994; Zheng *et al.*, 1994; Rueda *et al.*, 1995; Young *et al.*, 1997).

Ubiquitin is a highly conserved 76 amino acid polypeptide that covalently conjugates to cytoplasmic proteins and marks

them for subsequent proteolytic degradation. Ubiquitin also binds to histones and may regulate chromatin structure during transcription (Hershko, 1988) and thus has been immunochemically detected in both the cytoplasm and the nucleus (Haas and Bright, 1985). Ubiquitin upregulation has been associated with apoptosis: in the tobacco hawkmoth, *Manduca sexta*, the developmentally programmed cell death of intersegmental muscles is associated with a tenfold increase in ubiquitin (Haas *et al.*, 1996; Myer and Schwartz, 1996); the colonial sea squirt, *Botryllus schlosseri*, undergoes a cyclical cell death process characterized by apoptosis and upregulation of cytoplasmic ubiquitin immunoreactivity (Lauzon *et al.*, 1993); and murine myoblasts committed to apoptosis show increased expression of ubiquitin (Sandri *et al.*, 1997). Ubiquitin mRNA is upregulated and nuclear proteins are ubiquitinated in human lymphocytes induced to undergo apoptosis (Delic *et al.*, 1993). However, embryonic neurones induced to undergo apoptosis *in vitro* do not upregulate ubiquitin mRNA (D'Mello and Galli, 1993), which indicates that the role and subcellular localization of ubiquitin during apoptosis is not entirely clear.

Functional luteal regression in marmoset monkeys occurs 18–24 days after ovulation (Harding *et al.*, 1982), but the

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entire process of regression can take approximately 2 weeks from the beginning of functional regression to the completion of structural regression. It is possible to shorten the duration of functional and structural luteolysis in marmosets by inducing luteal regression with either PGF_{2α} (Summers *et al.*, 1985; Fraser *et al.*, 1995) or GnRH antagonist 9 days after ovulation (Hodges *et al.*, 1988). Functional regression is complete and structural regression is well advanced 24 h after treatment (on day 10 of the luteal phase).

The aims of this study were: (i) to examine the expression of ubiquitin in the corpora lutea of marmoset monkeys after induced luteolysis; (ii) to confirm previous reports of an increase in apoptosis during structural luteal regression; and (iii) to assess ubiquitin as an early indicator of apoptotic cell death.

Materials and Methods

Animals and treatments

Female marmoset monkeys (*Callithrix jacchus jacchus*) were housed at the MRC Reproductive Biology Unit Primate Centre as described by Hearn *et al.* (1975). All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986. Ovarian cycles were monitored by taking venous blood samples on alternate days and measuring the plasma progesterone concentration (Smith *et al.*, 1990). The follicular phase was defined as the period during which progesterone concentrations were 20 nmol l⁻¹ or less. Day 1 of the luteal phase was assumed to be the day on which the progesterone concentration rose above 32 nmol l⁻¹ and was followed by a sustained elevation in progesterone.

Animals were monitored before inclusion in the study to ensure ovarian cycles were normal. Ovaries were collected as described by Young *et al.* (1997). Briefly, animals were treated on day 9 of the luteal phase with either the GnRH antagonist antarelix (1 mg kg⁻¹ s.c.; Europeptides, Argenteuil) (Deghenghi *et al.*, 1993) or the PGF_{2α} analogue cloprostenol (1 µg i.m.; Planate, Coopers Animal Health Ltd, Crewe) (Summers *et al.*, 1985; Fraser *et al.*, 1995). Ovaries were collected 24 h after luteolytic treatment ($n = 6$ for both treatments) and were compared with ovaries obtained from untreated animals on day 10 of the luteal phase ($n = 7$). One additional animal was treated with GnRH antagonist on days 8 and 9 of the luteal phase and ovaries were collected on day 10.

Tissue collection and preparation

Animals were sedated with 100 µl ketamine hydrochloride (200 mg ml⁻¹; Parke-Davies Veterinary, Pontypool) administered i.m. and then killed with 400 µl Euthatal administered i.v. (100 mg sodium pentobarbitone ml⁻¹; Rhône Mérieux, Harlow). A terminal blood sample was taken before removing ovaries. Ovaries for immunocytochemical analysis were fixed in 4% (w/v) buffered paraformaldehyde for 24 h before embedding in paraffin wax according to standard

procedures. Sections (4 µm) were mounted on slides coated with poly-L-lysine (50 µg l⁻¹). Corpora lutea were dissected free of ovaries (day 10 of luteal phase controls, $n = 4$; 24 h after PGF_{2α} treatment, $n = 3$; 24 h after GnRH antagonist treatment, $n = 3$; and 48 h after GnRH antagonist treatment, $n = 1$) and snap-frozen in liquid nitrogen for DNA extraction and analysis of oligonucleosome formation. Liver samples were also collected and frozen for use as negative control tissues in the oligonucleosome study.

Immunocytochemistry

Paraformaldehyde fixed sections were rehydrated through graded alcohols to water then subjected to antigen retrieval by exposure to four 5 min cycles of microwave irradiation at 700 W in 0.01 mol sodium citrate l⁻¹ buffer (pH 6.0) as described by Shi *et al.* (1993). The sections were then cooled at room temperature for 20 min before washing in Tris-buffered saline (TBS, 0.05 mol Tris l⁻¹, 9 g NaCl l⁻¹). Primary rabbit anti-bovine ubiquitin polyclonal antibody (DAKO, High Wycombe) was diluted 1:50 in TBS and applied for 36 h at 4°C. Corresponding negative controls were treated with 1:50 normal rabbit serum (SAPU, Carlisle). After washing with TBS, secondary antibodies were applied in a blocking solution as follows: biotinylated goat anti-rabbit 1:500 (DAKO, High Wycombe) in 20% normal goat serum, 5% BSA (w/v) in TBS for 1 h at room temperature. Visualization was with avidin-biotin complex conjugated to alkaline phosphatase (Vector, Peterborough) made up according to the manufacturer's instructions and applied for 1 h at room temperature, followed by application of Fast Red stain made up to the manufacturer's instructions (Vector, Peterborough).

Ubiquitin quantification and statistics

Slide identification was obscured and independent assessment of qualitative differences between treatments was carried out by two observers. Quantitative analysis was also conducted blind using a ×40 objective lens. Six randomly selected fields of view of luteal tissue were scored for each animal and the mean number of ubiquitin-positive cells per ×40 field of view was calculated for each animal. Scoring more than five fields of view did not significantly alter the accumulative mean. Localization of ubiquitin to either the nucleus or the cytoplasm was also recorded. Mean numbers were calculated for each experimental group and expressed as mean ± SEM. A two-tailed paired Student's *t* test was applied to day 10 controls and to each of the two treatment groups. Significance was assigned at $P < 0.05$.

DNA extraction and oligonucleosome detection

Cellular DNA was extracted and the 3' ends were labelled using a method modified from Tilly and Hsueh (1993). Luteal tissue (approximately 20 mg) was homogenized in 400 µl buffer (0.1 mol NaCl l⁻¹, 0.3 mol Tris l⁻¹, 0.01 mol EDTA l⁻¹ and 0.2 mol sucrose l⁻¹, pH 8) with a handheld

electric homogenizer (Polytron PT 1200B92; Phillip Harris Co. Ltd, Glasgow) and then incubated at 65°C for 2–3 h with the addition of 25 µl of 10% (w/v) SDS and 25 µl of 0.01 mg proteinase-K µl⁻¹ (Boehringer Mannheim, Lewes). After incubation, samples were centrifuged at 5000 g for 20 min at 4°C. The supernatant was collected and nucleic acids were extracted first with phenol:chloroform:isoamyl alcohol (25:24:1) and subsequently with chloroform:isoamyl alcohol (24:1). DNA (15 µl) and the following reagents were used for 3' labelling: 15 µl of 5 × reaction buffer (1 mol potassium cacodylate l⁻¹, 0.125 mol Tris-Cl l⁻¹, 1.25 mg BSA ml⁻¹, pH 6.6), 5 µl of 25 mmol CoCl l⁻¹ and 1 µl terminal transferase (25 U µl⁻¹) (Boehringer Mannheim, Lewes). Radiolabelled dideoxynucleotide (2 µl of [α -³²P]ddATP (110 TBq mmol⁻¹), Amersham International, Amersham) was added before incubation at 37°C for 60 min. The reaction was terminated by the addition of 20 µl of 0.25 mol EDTA l⁻¹. Samples were loaded onto Sephadex G50 Nick Spin Columns (Pharmacia, Uppsala) and centrifuged at 500 g for 5 min to separate DNA-bound radionuclide from free radionuclide. Labelled DNA was subjected to electrophoresis on a 2% agarose gel for 2.5 h at 85 V using a 1 × TAE (40 mmol Tris-acetate l⁻¹, 1 mmol EDTA l⁻¹) running buffer. The gel was dried and exposed to Kodak X-Omat AR film for 2–12 h at -72°C. DNA extraction and labelling was conducted at least twice on each sample.

Progesterone assay

Progesterone concentrations were determined by radioimmunoassay as described by Smith *et al.* (1990). The sensitivity of the assay was 0.07 pmol per tube and the inter- and intra-assay coefficients of variation were 15 and 4%, respectively. The means of each group were expressed $\pm$ SEM and subjected to a two-tailed Student's *t* test with significance assigned at $P < 0.01$.

Results

On the day of ovary collection progesterone concentrations were at luteal phase values 311 ± 48 nmol l⁻¹ ($n = 3$) in all midluteal phase control animals but were significantly reduced to 18.33 ± 3.75 nmol l⁻¹ ($P < 0.01$, $n = 3$) and 36.35 ± 16.32 nmol l⁻¹ ($P < 0.01$, $n = 3$) after luteolytic treatment with PGF_{2 α} and induction of luteolysis with GnRH antagonist, respectively.

Low oligonucleosome formation was observed in one midluteal phase animal on day 10 (Fig. 1, lane 1), but apoptotic DNA fragmentation was absent from three other midluteal phase animals (results for two animals are shown in Fig. 1, lanes 2 and 3). Oligonucleosome fragmentation was present in all animals after induced regression with either GnRH antagonist (Fig. 1, lanes 4 and 5) or PGF_{2 α} (Fig. 1, lanes 6 and 7). Smearing was also observed, which is indicative of non-apoptotic DNA degradation. This was inconsistent in control samples, but non-apoptotic DNA fragmentation was always present in the corpora lutea of animals given luteolytic treatments. DNA fragmentation was not observed in the liver samples.

Luteolytic treatment with either PGF_{2 α} or GnRH antagonist caused changes in the morphological appearance of corpora lutea from regular organized tissue consisting of large rounded steroidogenic cells (Webley *et al.*, 1990; Fehrenbach *et al.*, 1995) embedded in a vascular network of capillaries and small blood vessels, to a disorganized irregular structure in which the vasculature was dilated and the parenchymal cells were oedematous with cytoplasmic vacuoles. Ubiquitin immunostaining was localized in the nuclei of steroidogenic cells (1.66 ± 0.66 cells per $\times 40$ field of view, $n = 3$, Figs 2a and 3) in midluteal phase corpora lutea on day 10, but was not found in the nuclei of luteal cells after luteolytic treatment (Fig. 3). Cytoplasmic ubiquitin immunoreactivity was apparent in 0.4 ± 0.3 midluteal phase cells per $\times 40$ field of view, and this was significantly increased to 12.24 ± 1.6 cells ($P < 0.02$, $n = 3$) per $\times 40$ field of view in PGF_{2 α} -treated animals (Figs 2c and 3). Ubiquitin immunoreactivity was not observed after induction of luteolysis with GnRH antagonist (Fig. 2d), nor was it found in negative control sections (Fig. 2b).

Discussion

This is the first report of cytoplasmic ubiquitin upregulation after PGF_{2 α} -induced luteolysis in a primate. GnRH antagonist-induced luteolysis did not cause an increase in ubiquitin expression. Oligonucleosome formation characteristic of apoptotic DNA fragmentation increased after induced luteal regression with both PGF_{2 α} and GnRH antagonist, which suggests that ubiquitin expression is not related to apoptosis in marmoset luteal regression.

Ubiquitin was found in the nuclei of a small number of midluteal phase steroidogenic cells, and it has previously been shown that $< 1\%$ of steroidogenic cells become 3'-labelled *in situ* during the midluteal phase (Young *et al.*, 1997). Therefore, nuclear ubiquitin may be related to apoptosis during the midluteal phase. However, it is also possible that it has a role in transcription and cell cycle protein regulation (Levinger and Varhavsky, 1982; Huang *et al.*, 1986). Ubiquitin was also found in the cytoplasm of parenchymal luteal cells 24 h after induction of luteolysis with PGF_{2 α} . The shift from nucleus to cytoplasm and the increase in the number of cells expressing ubiquitin suggests that ubiquitin has different roles before and after luteolysis, and may be involved in cytoplasmic proteolysis or protein regulation during PGF_{2 α} -induced luteal regression. Murdoch *et al.* (1996) reported an increase in luteal ubiquitin mRNA and protein as rapidly as 2 h after administration of a luteolytic dose of PGF_{2 α} to ewes. This observation suggests that ubiquitin is involved in functional luteal regression, since structural regression, as measured by total luteal mass, did not begin until at least 16 h after administration of PGF_{2 α} in this study. However, this does not preclude ubiquitin from involvement in structural regression. Ubiquitin immunoreactivity was not found in any animal subjected to GnRH antagonist-induced luteolysis. These data suggest that ubiquitin was not involved in structural regression in GnRH antagonist-treated animals, although it is possible that ubiquitin expression occurred earlier and was involved

Fig. 1. Autoradiograph of 3'-labelled DNA from marmoset corpora lutea subjected to electrophoresis on a 2% agarose gel. Lanes 1-3, midluteal phase controls; lanes 4 and 5, GnRH antagonist administered *in vivo* on day 9 of the luteal phase; lanes 6 and 7, PGF_{2α} administered *in vivo* on day 9 of the luteal phase. All corpora lutea were collected on day 10 of the luteal phase. Lower lanes show negative control liver samples corresponding to the animal in the lane above. Molecular weight markers are shown on the right hand side.

in functional luteal regression. Therefore, ubiquitin expression in luteal cells may be a response to prostaglandin because only cells with prostaglandin receptors expressed ubiquitin in ovine corpora lutea (Murdoch *et al.*, 1996). Furthermore, luteal ubiquitin expression may not be specific for luteolysis but forms part of a response to cellular stress in which ubiquitin is coexpressed with heat shock protein 70 (Murdoch *et al.*, 1996), which is upregulated after PGF_{2α}-induced regression in rats (Khanna *et al.*, 1995).

Apoptosis has been shown to occur during structural luteolysis (Murdoch, 1995). The extent of oligonucleosome formation observed in marmoset corpora lutea in the present study confirms previous observations that showed an increase in apoptosis after luteolytic treatment (Young *et al.*, 1997). The small amount of oligonucleosome formation in midluteal corpora lutea observed in the present study is in agreement with the small amount of *in situ* 3' labelling previously observed in these animals, in which < 1% of cells undergo apoptosis during the midluteal phase (Young *et al.*,

1997). Luteolytic treatment with PGF_{2α} or GnRH antagonist increased apoptotic cell numbers to $8.04 \pm 1\%$ and $7.4 \pm 1.5\%$, respectively, in 3'-labelled sections, and there was a corresponding increase in apoptotic oligonucleosome formation 24 h after induction of luteolysis with both PGF_{2α} and GnRH antagonist. Oligonucleosome formation 24 h after induction of luteolysis with PGF_{2α} has also been reported in bovine (Juengel *et al.*, 1993; Rueda *et al.*, 1995), ovine (Kennny *et al.*, 1994), and rabbit (Dharmarajan *et al.*, 1984) corpora lutea, but there has been no other demonstration of oligonucleosome formation after a luteolytic dose of GnRH antagonist. Non-specific DNA degradation has been associated with necrotic cell death, and parenchymal luteal cells in the present study displayed some characteristics of necrosis after luteolytic treatment (that is, DNA margination, formation of cytoplasmic vacuoles and oedematous swelling). The non-specific DNA fragmentation observed after induced luteal regression may be attributable to this particular form of cell death.

Fig. 2. Immunohistological detection of ubiquitin in 4 µm sections of paraffin wax-embedded marmoset ovaries using the avidin–biotin alkaline-phosphatase method. Dark grey to black areas represent positive immunoreactivity. (a) Ubiquitin-positive nucleus and cytoplasm of steroidogenic cells in a corpus luteum on day 10 of the luteal phase. (b) Negative control section for (a) with primary ubiquitin antibody omitted; no positive immunoreactivity. (c) Ubiquitin-positive cytoplasm in steroidogenic cells in a regressed corpus luteum on day 10 of the luteal phase 24 h after administration of $\text{PGF}_{2\alpha}$ analogue. (d) No ubiquitin immunoreactivity in regressed corpora lutea on day 10 of the luteal phase 24 h after administration of GnRH antagonist. Scale bars represent 20 µm.

Fig. 3. Mean ($\pm$ SEM) number of luteal cells exhibiting either nuclear (□) or cytoplasmic (■) ubiquitin immunoreactivity per $\times 40$ field of view in paraformaldehyde-fixed sections of marmoset ovaries. Ovaries were collected on day 10 of the luteal phase from control animals ($n = 3$) and from animals treated 24 h previously with a $\text{PGF}_{2\alpha}$ analogue ($n = 3$). Asterisk indicates value is significantly different ($P < 0.02$) from corresponding midluteal phase control.

Ubiquitin has been shown to be upregulated during apoptosis in a number of systems (Delic *et al.*, 1993; Lauzon *et al.*, 1993; Haas *et al.*, 1996; Myer and Schwartz, 1996; Sandri *et al.*, 1997). However, there does not seem to be a relationship between ubiquitin expression and apoptosis during luteal regression in marmoset monkeys. Cells expressing ubiquitin in $\text{PGF}_{2\alpha}$ -treated animals did not have the same morphology as ^3H -labelled cells in serial sections; death of ubiquitin-positive cells appeared to be by a non-apoptotic autophagocytotic pathway. In addition, apoptosis has been demonstrated 24 h after administration of GnRH antagonist (Young *et al.*, 1997) but this is not coincident with ubiquitin expression in these animals.

In conclusion, ubiquitin is expressed in the nuclei of a small number of midluteal phase steroidogenic cells and in the cytoplasm of steroidogenic cells after $\text{PGF}_{2\alpha}$ -induced luteal regression, but is not expressed in luteal cells after a luteolytic dose of GnRH antagonist. Induced luteolysis with both $\text{PGF}_{2\alpha}$ and GnRH antagonist is associated with an increase in apoptosis, but ubiquitin expression is not an indicator of apoptosis in the corpus luteum of marmoset monkeys.

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