Progesterone on an oestrogen background enhances prolactin-induced apoptosis in regressing corpora lutea in the cyclic rat: possible involvement of luteal endothelial cell progesterone receptors

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Abstract

Preovulatory surges of both prolactin (PRL) and progesterone have been suggested to be necessary for the induction of apoptosis in the regressing corpus luteum of the cyclic rat. The aim of these experiments was to study whether the administration of PRL and/or progesterone on the morning of pro-oestrus reproduces the regressive changes that happen in the cyclic corpus luteum (CL) during the transition from pro-oestrus to oestrus, and to analyse the temporal relationships between two characteristic features of structural luteolysis (luteal cell apoptosis and accumulation of macrophages). Cyclic rats (treated at 0900 h with an LHRH antagonist to block LH secretion) were injected at 1000 h with PRL and progesterone and killed at 0, 30, 60, 90 and 180 min after treatment. The number of apoptotic cells increased progressively from 60 min after treatment onward in hormone-treated rats, whereas the number of macrophages did not change throughout the period of time considered. Rats injected with PRL plus progesterone showed significantly greater numbers of apoptotic cells than those injected with PRL alone. The luteolytic effects of progesterone were in keeping with the presence of luteal endothelial cells showing progesterone receptor (PR) immunoreactivity in pro-oestrus. Treatment of rats during dioestrus and pro-oestrus with the specific antioestrogens LY117018 and RU58668 decreased the luteolytic effects of PRL and progesterone and the number of luteal endothelial cells immunostained for PR. These results strongly suggest that the preovulatory PRL surge and the preovulatory increase in progesterone together trigger structural regression of the corpus luteum. This seems to be dependent on oestrogen-driven cyclic changes in PRs in luteal endothelial cells.

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Introduction

In the rat, as in some of its close relatives, prolactin (PRL) has two effects on the corpus luteum (CL). One is to maintain the ability of the CL to secrete progesterone; this is referred to as the luteotrophic effect. The other causes rapid regression of CL that are unable to respond to the luteotrophic action of PRL; this effect is referred to as the luteolytic effect (Rothchild 1965, 1981). The luteolytic effect of PRL includes the induction of luteal cell death and invasive accumulation of macrophages (Bowen *et al.* 1996, Gaytán *et al.* 1998), which lead to the regression in size of the CL (Sánchez-Criado *et al.* 1987). Although the mechanisms involved are not clear, some studies suggest that the luteolytic actions of PRL are ultimately mediated by prostaglandins, because PRL-induced CL regression is attenuated by pharmacological inhibition of prostaglandin synthesis (Sánchez-Criado *et al.* 1987). Macrophages recruited into the regressing CL could also mediate the luteolytic actions of PRL through the release of cytotoxic cytokines and/or reactive oxygen species (Bagavandoss *et al.* 1990, Bowen *et al.* 1996, Towson *et al.* 1996).

In a previous study (Gaytán *et al.* 1998), we proposed that both PRL and progesterone were necessary on the day of pro-oestrus for the induction of apoptosis in the regressing CL of the cycle. This was suggested by the inhibition of apoptosis during the transition pro-oestrus–oestrus in rats lacking progesterone actions as a result of treatment with the progesterone antagonists RU486 and ZK299, whereas the accumulation of macrophages seems

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to be dependent exclusively on the PRL surge, because it was only blocked by treatment with the dopaminergic agonist CB154 (Gaytán *et al.* 1997, 1998). However, several questions about the roles of PRL and progesterone in CL regression remain unanswered. First, as evidence for a role of progesterone in luteolysis was derived via a pharmacological approach, is possible that the inhibition of PRL-induced apoptosis in RU486- or ZK299-treated rats was due, at least in part, to the blockade of ligand-independent activation of progesterone receptor (PR) by antiprogestins (Weigel & Zhang 1998, Canni & Picard 1999, Sánchez-Criado *et al.* 1999) rather than to the lack of progesterone action. Secondly, although the accumulation of macrophages in the absence of apoptosis in antiprogestin-treated rats suggests that these cells are not the effectors of apoptosis, it cannot be ruled out that a second signal, for instance progesterone, which is known to modify some CL macrophage functions (Sugino *et al.* 1996), could be necessary to activate PRL-recruited macrophages to a cytocidal state. Thirdly, as PR seem to be absent from the rat steroidogenic luteal cells (Natraj & Richards 1993), it is not clear whether the proposed luteolytic effects of progesterone are mediated by activation of PR and, if so, what cell type in the CL expresses PR.

The experiments we describe here were directed towards obtaining answers to these questions. With this aim, we studied whether the administration of PRL and/or progesterone on the morning of pro-oestrus and after secretion of endogenous luteinizing hormone (LH) and progesterone had been blocked, would reproduce the regressive changes that take place in the CL of the cycle during the transition from pro-oestrus to oestrus. We also analysed the temporal relationships between apoptosis and accumulation of macrophages, the immunolocalization of PR in the CL throughout the oestrous cycle, and the involvement of the oestrogen background in progesterone-induced apoptosis.

Materials and Methods

Animals and chemicals

Female cyclic rats of the Wistar strain (250 g on average) were used. The animals were maintained under controlled conditions of light (14 h light : 10 h darkness; lights on at 0500 h) and temperature (21 °C) and had free access to rat chow and tap water. The stage of the cycle was checked daily by the examination of vaginal smears. Only rats showing at least two consecutive 4-day cycles were used.

Ovine PRL (oPRL-18) was obtained from the NIADDK (Baltimore, MD, USA). Progesterone was obtained from Sigma Chemical Company (St Louis, MO, USA). The LH releasing hormone (LHRH) antagonist (LHRHa) used was ORG.30276 (Ac-D-*p*-Cl-Phe-D-*p*-Cl-Phe-D-Trp-Ser-Tyr-D-Arg-Leu-Arg-Pro-D-Ala-$NH_2{\cdot}CH_3{\cdot}COOH$; Organon, Oss, The Netherlands). The antioestrogens LY117018-HCl and RU58668 were obtained from Lilly SA (Madrid, Spain), and from Roussel-Uclaf (Romainville, France), respectively.

Experimental design

Experiment 1 This experiment was performed to analyse the temporal relationships between the appearance of apoptotic cells and the accumulation of macrophages. Cyclic rats were injected s.c. on the morning of pro-oestrus (0900 h) with LHRHa (1 mg/0·2 ml saline). This dose has been previously reported to be effective in suppressing the LH surge on the afternoon of pro-oestrus (Sánchez-Criado *et al.* 1993), and hence LH-driven secretion of progesterone. At 1000 h the animals were injected s.c. with PRL (250 μg/rat, in 0·03 M $NaHCO_3$, 0·15 M NaCl, 0·5% BSA pH 9·5) and progesterone (5 mg/rat, in 70% ethanol) or vehicles. These doses were selected on the basis of the findings of previous studies (Tébar *et al.* 1995, Gaytán *et al.* 1997). Five animals per time point were killed immediately before treatment (time 0) and at 30, 60, 90 and 180 min after treatment. Vehicle-injected rats were killed at 180 min. Trunk blood was collected for determination of progesterone and the ovaries were fixed for 24 h in Bouin–Hollande fluid at room temperature or in 4% paraformaldehyde (PFA) at 4 °C and processed for paraffin embedding. The number of apoptotic cells and macrophages was counted. In a previous study we showed that no differences existed between apoptotic cell counts based on morphological criteria and those based on 3′-end labelling (TUNEL method) in the regressing CL of cyclic rats (Gaytán *et al.* 1998). The TUNEL method was applied to some ovaries at 180 min after treatment, following previously described procedures (Gaytán *et al.* 1998), to demonstrate that cell death induced by PRL plus progesterone treatment in pro-oestrus was equivalent to that found in cyclic rats.

Experiment 2 This was performed to compare the luteolytic effects of PRL in the presence or the absence of progesterone. Cyclic rats treated with LHRH antagonist as in experiment 1 were injected s.c. at 1000 h with PRL (250 μg), progesterone (5 mg), PRL plus progesterone (250 μg and 5 mg respectively) or vehicles and sacrificed at 180 min. Trunk blood was collected and stored until assayed for determination of progesterone. In these animals, the ovaries were fixed and processed as in experiment 1 and the number of apoptotic cells was counted.

Experiment 3 Cyclic rats were used for the immunolocalization of PR in the CL. Three rats per day of the cycle were sacrificed at 1100 h and an additional group of three rats on the evening (2100 h) of pro-oestrus. The lungs, heart, spleen, uterus and ovaries were dissected and

fixed for 24 h in Bouin–Hollande fluid at room temperature or in 4% PFA at 4 °C and processed for paraffin embedding. The percentage of nuclei immunostained for PR were counted.

Experiment 4 To test whether the oestrogen background in dioestrus–pro-oestrus was involved in the luteolytic effects of progesterone, cycling rats were injected s.c. in dioestrus (0900 h) and pro-oestrus (0900 h) with two specific antioestrogens lacking agonist activity – LY117018 (4 mg/rat) (Clemens *et al.* 1983, Tébar *et al.* 1998) and RU58668 (0·5 mg/rat) (Vagell & McGinnins 1997) – or with vehicle (200 µl olive oil). Thereafter, these rats were injected with LHRHa (0900 h) to block LH secretion. On the morning of pro-oestrus (1000 h) these rats were injected with PRL and progesterone as in previous experiments and sacrificed at 180 min after treatment with PRL plus progesterone. The ovaries were processed as in previous experiments to study the presence of apoptotic cells in regressing corpora lutea. In addition, PR was immunolocalized in ovaries of rats injected with LY117018 or vehicle (three rats per group) before treatment with PRL plus progesterone.

Immunohistochemical assay of PR

For the immunohistochemical assay, the monoclonal antibody PRI0A9 (Immunotech, Marseille, France) raised against recombinant hormone-binding domain (922AG-MVKPLLFHKK933) of human PR conjugated to BSA was used, with the avidin–biotin peroxidase complex (ABC) technique. This antibody reacts with the C-terminal domain of human PR.

Dewaxed and rehydrated Bouin-Hollande-fixed paraffin-embedded sections were subjected to a high-temperature antigen unmasking technique by incubation with boiling citric acid buffer pH 6·0 for 10 min. After cooling at room temperature for 20 min, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 min at room temperature, tissue sections were rinsed with PBS pH 7·6 (3 × 10 min) and incubated with 10% normal goat serum (Master Diagnostica, Córdoba, Spain) in PBS. The specific primary antibody, diluted 1:20, was incubated overnight at 18 °C, and after three washes in PBS of 10 min each, a biotinylated goat anti-mouse immunoglobulin antibody (Dako, Glostrup, Denmark) diluted 1:20 was applied for 30 min at room temperature. After PBS rinsing, sections were treated for 1 h at room temperature with the ABC complex (Vector Laboratories Inc., Burlingame, CA, USA) diluted 1:50 in PBS, rinsed with Tris buffer saline (TBS) pH 7·6, incubated with the chromogen 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St Louis, MO, USA) diluted 0·035% in TBS for 1 min, rinsed in tap water, counterstained with Mayer's haematoxylin, dehydrated and mounted.

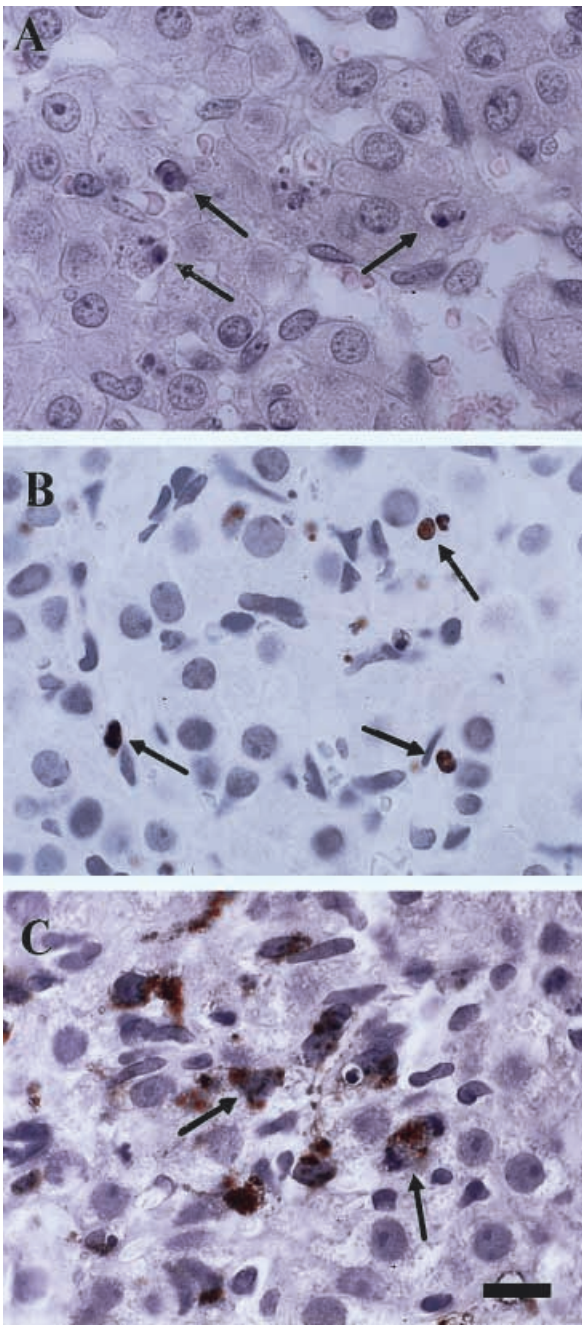

Figure 1 Micrographs of CL on the morning of pro-oestrus 180 min after treatment with PRL (250 µg) plus progesterone (5 mg), stained with haematoxylin and eosin (A) or immunostained with the *in situ* 3′-end labelling (TUNEL) method (B). Some apoptotic cells are indicated by arrows. (C) Macrophages (arrows) immunostained with ED1 antibody. All micrographs are printed at the same magnification. Scale bar (applicable to all three micrographs) represents 20 µm.

Tissue sections of the uterus at oestrus, metoestrus, dioestrus and pro-oestrus were run as positive controls in

every assay; sections of heart, lung and spleen were used as negative controls in the standardization of the technique. Pieces of the lung, heart, spleen (negative controls), uterus (positive control) and the ovaries were processed together, embedded in the same block and subjected to immunohistochemical procedures on the same poly-L-lysine-coated slide. The substitution of the specific primary antibody by mouse ascitic fluid diluted 1:20 in tissue sections of the samples under study was used as negative control in every assay.

Cell counting

The number of apoptotic cells was counted in Bouin–Hollande-fixed tissues stained with haematoxylin and eosin and macrophages in PFA-fixed tissues immunostained for ED1 antibodies in five animals per group, following previously described procedures (Gaytán *et al.* 1998). Briefly, apoptotic cells showed specific morphological features consisting of chromatin condensation and eosinophilic shrunken cytoplasm, followed by fragmentation of the nuclei into discrete chromatin masses. Use of the TUNEL method confirmed that these cells contained fragmented DNA. Macrophages were recognized in PFA-fixed immunostained sections with ED1 antibody, which is specific for rat macrophages (Damoiseaux *et al.* 1994), and has been used previously for the identification of macrophages in the rat CL (Bowen *et al.* 1996, Gaytán *et al.* 1998). At least three regressing corpora lutea of the current cycle were studied per rat. In each CL three equatorial sections (at least 25 μm apart to avoid counting repetitions) were systematically screened and the number of apoptotic cells or macrophages expressed per area unit of CL section. In sections stained for PR immunohistochemistry (three animals per day of the cycle), only nuclear immunostaining was considered. The percentage of luteal cells with immunostained nuclei was found with respect to the total number of non-steroidogenic cells, following the same sampling rules as for macrophages or apoptotic cells.

Determination of progesterone

Serum progesterone concentrations were determined using a commercially obtained kit (Diagnostic Product Corporation, Los Angeles, CA, USA). Assay sensitivity was 10 pg/tube and the intra-assay coefficient of variation was 6%.

Statistical analysis

Statistical analyses were performed by ANOVA followed by Tukey's test for multiple comparison among means or Student's *t*-test when only two means required comparison. Data are given as the mean ± S.E.M., for $n=5$ for apoptotic cells and macrophages or serum progesterone concentrations, and $n=3$ for the percentage of cells immunostained for PR.

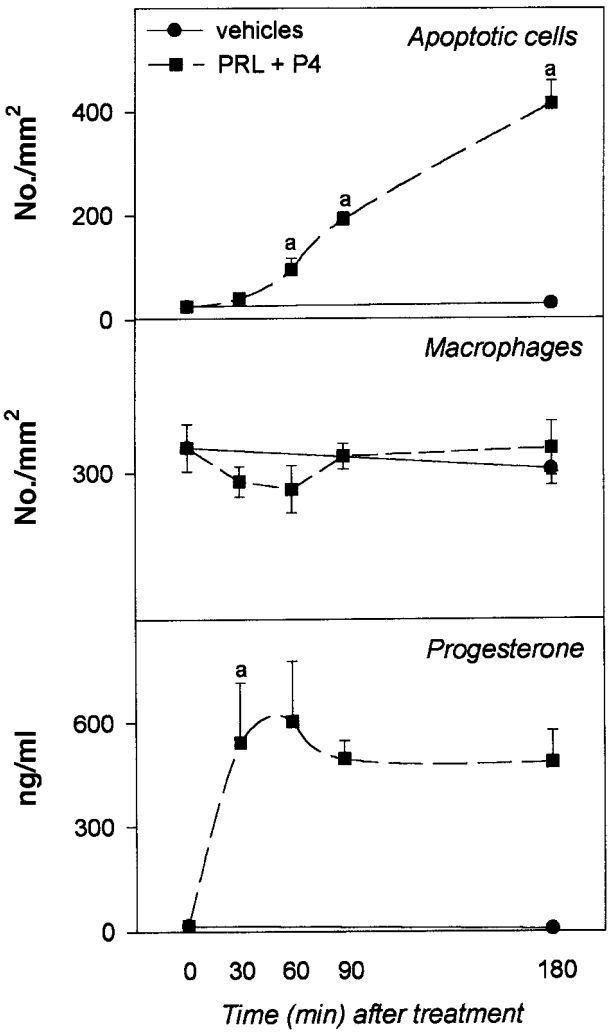

Figure 2 Time course of the changes in the number of apoptotic cells, macrophages and serum progesterone concentrations after administration of PRL plus progesterone P4; (see legend to Fig. 1 for details) on the morning (1000 h) of pro-oestrus in cyclic rats. [a]$P<0{\cdot}01$ compared with the previous time point (ANOVA and Tukey's test for $n=5$).

Results

Time course of the luteolytic effects of PRL plus progesterone

On the morning of pro-oestrus, morphological signs of structural luteolysis were not observed in the CL of control rats. Apoptotic cells were only occasionally found and macrophages, although relatively abundant, were present in numbers similar to those found at dioestrus. After

treatment with PRL plus progesterone, apoptotic cells were frequently observed, and were abundant at 180 min after treatment (Fig. 1A). Dying cells were immunostained with the TUNEL method (Fig. 1B). No changes in the macrophage population were observed (Fig. 1C). Quantitative data are shown in Fig. 2. The number of apoptotic cells was increased significantly ($P<0{\cdot}01$) from 60 min after treatment onward, whereas the number of macrophages did not change during the period of time considered. Serum progesterone concentrations were increased significantly ($P<0{\cdot}01$) from 30 to 180 min after treatment in progesterone-injected animals. In rats injected with vehicles, progesterone concentrations remained at basal values.

Luteolytic effects of PRL and/or progesterone

Rats treated with PRL plus progesterone showed significantly ($P<0{\cdot}01$) greater (about 60%) numbers of apoptotic cells than rats treated with PRL alone, whereas the number of apoptotic cells in rats injected with progesterone alone was not different from that found in vehicle-injected rats (Fig. 3). Macrophage numbers did not change after any treatment. Serum progesterone concentrations were increased in animals injected with progesterone. In rats injected with PRL alone, serum progesterone concentrations ($4{\cdot}95 \pm 0{\cdot}46$ ng/ml, $n=5$) were not different from those found in vehicle-injected rats ($6{\cdot}78 \pm 0{\cdot}83$ ng/ml, $n=5$).

Immunolocalization of PR in cyclic rats

In positive control tissues (uterus), smooth muscle cells in the myometrium stained positively for PR in all stages of the cycle (Fig. 4A). Epithelial cells lining the uterine cavity and the endometrial glands were positive in met-oestrus and dioestrus and negative in pro-oestrus and oestrus. In the endometrial connective tissue, both positive and negative cell nuclei were found throughout the cycle (Fig. 4A). The endothelium of the blood vessels was always negative (Fig. 4A), whereas perivascular and stromal fibroblast-like cells were positive. Changes in the intensity of the staining among the different days of the cycle, and for the same cell type, were not considered because such changes were found even between sections of the same sample and were therefore considered to be incidental.

In the CL, PR immunostaining was exclusively found in the nuclei of non-steroidogenic cells, although immunoreactivity was fainter than in uterine cells. Almost all immunostained cells were lining blood capillaries and were, therefore, easily identifiable as endothelial cells (Fig. 4B, E). Immunostained cells were relatively abundant at pro-oestrus (Fig. 4B) and scarce during the rest of the cycle. Steroidogenic luteal cells (recognizable by their large cytoplasm and round nuclei) were always negative. On the evening of pro-oestrus (2100 h), the granulosa cells of preovulatory follicles showed a faint immunostaining (Fig. 4C), whereas no immunostaining was present in small or large atretic follicles (Fig. 4D). The proportions of cells immunostained for PR with respect to the number of non-steroidogenic luteal cells were $32{\cdot}0 \pm 8{\cdot}8\%$ and $5{\cdot}6 \pm 3{\cdot}3\%$ in pro-oestrus and oestrus respectively (mean ± S.E.M. for $n=3$) and less than 2% during the dioestrous phase. However, during oestrus, evaluation of immunostained cells was difficult because of the presence of abundant apoptotic cells. Immunostained cells were not found in negative control (lung, spleen or heart) sections, or when the first antibody was replaced by non-immune serum.

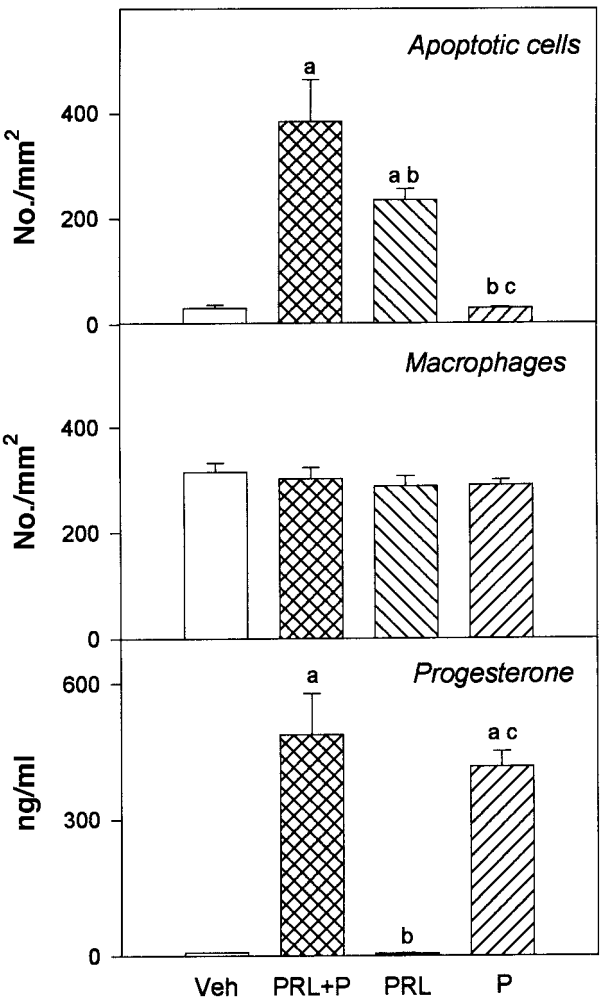

Figure 3 Effects of the administration of PRL plus progesterone (PRL+P), PRL, progesterone (P) or vehicle (Veh) on the morning of pro-oestrus, 180 min after treatment (see legend to Fig. 1 for details) on the number of apoptotic cells, macrophages and serum progesterone concentrations. Bars lacking common superscripts are significantly ($P<0{\cdot}01$) different (ANOVA and Tukey's test for $n=5$).

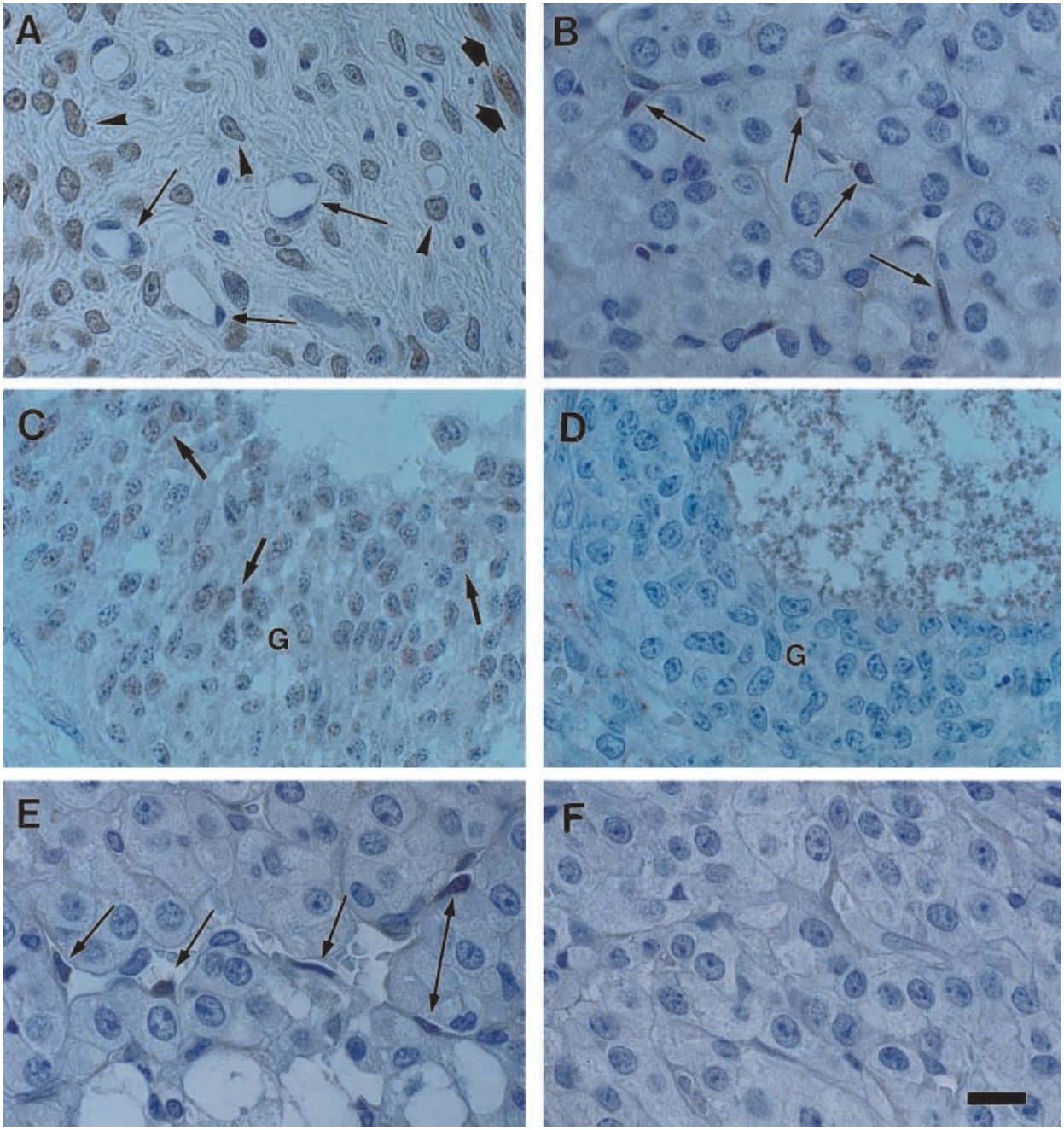

Figure 4 Micrographs of tissue sections immunostained for progesterone receptor. (A) Uterus at 1100 h on the day of pro-oestrus. Immunostained nuclei corresponded to stromal endometrial (arrowheads) and myometrial (short arrows) cells, whereas endothelial cells (long arrows) were negative for PR. (B) CL of the current cycle at 1100 h on the day of pro-oestrus. Some immunostained endothelial cells are indicated by arrows. (C), (D) Follicles on the evening (2100 h) of pro-oestrus. In healthy preovulatory follicles (C), granulosa cells (G) showed a faint immunostaining (some of them are indicated by arrows), whereas immunostaining was not found in the granulosa (G) of atretic follicles (D). (E), (F) Sections from CL on the morning of pro-oestrus (1000 h) treated with vehicle (E) or the antioestrogen LY117018 (F). Immunostained endothelial cells (arrows) were relatively abundant in vehicle-treated rats and extremely scarce in antiestrogen-treated rats. All sections shown here were counterstained with haematoxylin. All micrographs are printed at the same magnification. Scale bar (applicable to all six micrographs) represents 20 μm.

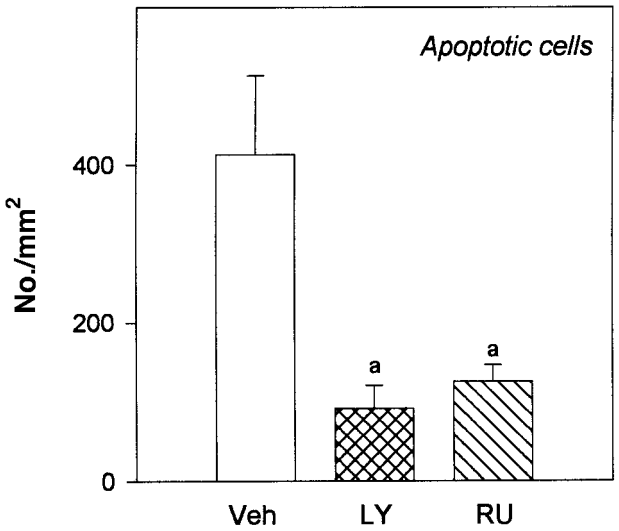

Figure 5 Number of apoptotic cells at 180 min after treatment with PRL plus progesterone (see legend to Fig. 1 for details) in rats treated with vehicle (Veh) or the antioestrogens LY117018 (LY) or RU58668 (RU). Bars lacking common superscripts are significantly ($P<0{\cdot}01$) different (ANOVA and Tukey's test for $n=5$).

Effects of oestrogen antagonist treatment

On the morning of pro-oestrus, vehicle-injected rats showed uterine ballooning and increasing numbers of eosinophil leucocytes in the myometrium and basal endometrium. Rats treated with antioestrogens showed morphological signs of oestrogen deprivation in the uterus, such as absence of ballooning and scarcity of eosinophil leucocytes. In these animals, the number of apoptotic cells in the regressing CL after administration of PRL and progesterone was greatly decreased. Quantitative data on the number of apoptotic cells are presented in Fig. 5. Antioestrogen-treated rats showed a 82% decrease in the number of apoptotic cells. In rats treated with the antioestrogen LY117018, cells immunostained for PR (Fig. 4F) were extremely scarce. The proportion of non-steroidogenic luteal cells immunostained for PR was $6{\cdot}8 \pm 6{\cdot}6\%$ compared with $29{\cdot}8 \pm 4{\cdot}5\%$ in vehicle-injected rats (mean ± S.E.M., $n=3$; $P<0{\cdot}01$, Student's t-test).

Discussion

The first signal for structural luteolysis in the cyclic rat seems to be the induction of apoptosis (Gaytán *et al.* 1998, Bowen *et al.* 1999) and the accumulation of macrophages in the regressing CL from late pro-oestrus to early oestrus (Gaytán *et al.* 1998) in response to the pro-oestrous afternoon preovulatory hormone surges. This couples ovulation and development of new luteal tissue to the structural regression of the previous generation of arrested CL. The administration of PRL plus progesterone on the morning of pro-oestrus to rats in which the endogenous secretion of gonadotrophins (and hence of LH-driven follicular progesterone secretion) has been suppressed induced luteal cell death in a manner similar to that found in cyclic rats during the transition from pro-oestrus to oestrus. Dying luteal cells showed the morphological signs of apoptosis and contained fragmented DNA as indicated by *in situ* 3′-end labelling. This validated the model for the study of the regulation of apoptosis in the regressing CL of the rat.

The induction of apoptosis in luteal cells in the absence of the classical accumulation of macrophages suggested that macrophages may not be the effectors of luteal cell death and that these events are independent and can be dissociated. In a previous study (Gaytán *et al.* 1998) we showed that accumulation of macrophages in the absence of luteal cell death occurred in antiprogestin-treated rats. Conversely, in this study, large numbers of apoptotic cells without any changes in the macrophage population were present at short times after PRL treatment. This agrees with previous studies in different species that suggested that the mere presence of increased numbers of macrophages is not enough to induce luteal cell death (Naftalin *et al.* 1997, Gaytán *et al.* 1998).

The administration of PRL plus progesterone was more effective in inducing luteal cell apoptosis than the administration of PRL alone. This supported the hypothesis of a role for progesterone in structural luteolysis (Gaytán *et al.* 1998). However, abundant apoptotic cells were found in rats treated exclusively with PRL whereas, in rats treated with progesterone antagonists, apoptotic cells were virtually absent (Gaytán *et al.* 1998). This could be explained by the action of basal concentrations of progesterone in LHRHa- and PRL-treated animals, whereas the model of antiprogestin-treated rats is, theoretically, equivalent to the complete absence of progesterone action.

Progesterone has been found to suppress apoptosis in many target tissues, such as the uterine epithelium (Rotello *et al.* 1992), mammary gland (Feng *et al.* 1995), and in granulosa cells of growing follicles (Peluso 1997), probably through classical nuclear receptors. Furthermore, several authors have suggested the existence of luteotrophic or antiluteolytic actions of progesterone in the CL (Rothchild 1981, Telleria & Deis 1994). These studies have examined the temporal relationships between the decrease in progesterone secretion (functional regression of the CL) and the structural luteolysis. As morphological signs of regression in the CL are preceded by luteal arrest (loss of the ability to make progesterone), the existence of cause-and-effect relationships is suggestive. However, no conclusive data on direct effects of decreased progesterone concentrations in the induction of structural CL regression are available. Nevertheless, the proposed luteolytic role of progesterone on pro-oestrus does not preclude the

existence of a luteotrophic role for this steroid in developing (Rothchild 1965, 1981, Fanjul *et al.* 1983, Telleria & Deis 1994) and functioning (Telleria & Deis 1994) CL. This autocrine stimulatory role of progesterone on its own secretion seems to be exerted, in an oestrogen-dependent maner (Telleria & Deis 1994), on the CL through genomic (Natraj & Richards 1993) or non-genomic (Rothchild 1996, Peluso 1997) mechanisms.

The presence of classical nuclear PR in the ovary shows interspecies variations (Ohta *et al.* 1993, Peluso 1997, Hild-Petito *et al.* 1988). In the rat, PR is transitorily expressed in granulosa cells of preovulatory follicles after the LH surge but is undetectable in luteal steroidogenic cells (Natraj & Richards 1993). The existence of a role for progesterone in enhancing the luteolytic actions of PRL on a non-functional CL, and the inhibition of this effect by blocking PR activation with RU486 or ZK299 (Gaytán *et al.* 1998), strongly suggested that this endocrine action of progesterone is mediated through interaction with luteal PR. PR have been found in monkey (Duffy *et al.* 1997, Hild-Petito *et al.* 1988) and human (Iwai *et al.* 1990) CL. However, several studies did not find PR mRNA in rat CL (Park & Mayo 1991, Park-Sarge *et al.* 1995). In the present study a faint but consistent immunoreactivity to PR was observed in the nuclei of non-steroiodogenic luteal cells. Almost all immunostained cells could be easily identified as endothelial cells. The number of immunostained cells that could not be accurately identified, because the blood vessel lumen was not present, was very minimal. This seemed to indicate that PR are exclusively expressed in luteal endothelial cells, although further characterization of these cells would be of interest. Several lines of evidence support that this immunostaining was specific: i) the presence of cells with immunostained nuclei in uterine tissues expressing PR (Ohta *et al.* 1993); ii) the presence of a similar immunostaining in the granulosa cells of healthy preovulatory (but not in small or atretic) follicles at 2100 h on the day of pro-oestrus after the LH surge, which agrees with the transient expression of PR in preovulatory follicles reported previously (Park & Mayo 1991, Natraj & Richards 1993); iii) the existence of cyclic changes in the number of immunostained cells, which were more abundant at pro-oestrus, when the administration of progesterone was effective in enhancing the luteolytic effects of PRL; iv) the absence of immunostaining in the endothelial cells of other progesterone target tissues such as the endometrium; and v) the decrease in the number of immunostained cells in antioestrogen-treated rats that also showed decreased numbers of apoptotic cells after treatment with PRL plus progesterone. It should be considered that only a small part of the CL (about 30% of all non-steroidogenic cells) expressed PR. This could explain the difficulty in detecting PR mRNA that was experienced in previous studies (reviewed in Peluso 1997). Comparatively, the amount of granulosa cells in preovulatory follicles expressing PR on the evening of pro-oestrus is considerably greater than that of endothelial cells expressing PR in the regressing CL.

Previous studies have reported that PR in the granulosa cells of preovulatory follicles are induced by the LH surge during the afternoon of pro-oestrus (Natraj & Richards 1993). In this study, the expression of PR in luteal endothelial cells on the morning of pro-oestrus in LHRHa-treated rats could not have been induced by the LH surge. The expression of PR in the CL reported here seemed to be related to the circulating oestrogen concentrations (Smith *et al.* 1975). Oestrogens secreted during the 24-h period preceding 1000 h pro-oestrus are responsible for the pro-oestrous surges of PRL and LH (and hence pro-oestrous progesterone) (Smith *et al.* 1975), and for uterine ballooning and accumulation of eosinophil leukocytes (Freeman *et al.* 1989, Tchernitchin *et al.* 1989). The results of the current study suggest that oestrogens induce the expression of PR in luteal endothelial cells in pro-oestrus. This could explain the inhibition of progesterone-induced apoptosis in antioestrogen-treated animals, an inhibition similar in magnitude to that found after blocking PR in pro-oestrus (Gaytán *et al.* 1998). Cyclic changes in oestradiol concentrations seem to be the main (although indirect) regulators of structural luteolysis in the cyclic CL. An increase in oestradiol during dioestrus–pro-oestrus induces the preovulatory LH surge (which in turn induces pro-oestrous afternoon progesterone secretion) (Neill *et al.* 1971) and the preovulatory PRL surge (Freeman *et al.* 1989). In addition, oestradiol induces the expression of PR in luteal endothelial cells. Both PRL and progesterone (through interaction with PR in endothelial cells) seem to be the main inducers of apoptosis from late pro-oestrus to early oestrus in the cyclic CL undergoing luteal arrest.

As PRs have not been found in steroidogenic luteal cells in the rat, progesterone and its antagonists (Gaytán *et al.* 1998, this study) may act on some other cell type within the regressing CL. This agrees with the presence of immunoreactive PR in luteal endothelial cells and reinforces the idea that blood vessels have a pivotal role in CL function and regression (Meidan & Girsh 1997). Changes in the luteal microvasculature could be an early event in luteolysis that may trigger and/or accelerate CL regression (Niswender *et al.* 1976, Sawyer *et al.* 1990). Both stromal and steroidogenic apoptotic cells were observed in the regressing CL (Gaytán *et al.* 1998, this study), although the incidence of cell death in each cell type was not analysed. The presence of steroidogenic apoptotic cells, together with the apparent absence of PR in these cells, suggest that paracrine interactions are involved in the induction of apoptosis in the rat CL.

In summary, this study has provided evidence for a role for progesterone secreted on the afternoon of pro-oestrus in structural luteolysis, probably through interaction with oestrogen-inducible PR in luteal endothelial cells. The ultimate mechanisms involved in the induction of cell death by PRL and progesterone remain to be determined.

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