Progesterone is a cell death suppressor that downregulates Fas expression in rat corpus luteum

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Abstract In female rats, apoptotic cell death in the corpus luteum is induced by the prolactin (PRL) surge occurring in the proestrous afternoon during the estrous cycle. We have previously shown that this luteolytic action of PRL is mediated by the Fas/Fas ligand (FasL) system. During pregnancy or pseudopregnancy, apoptosis does not occur in the corpus luteum. Progesterone (P_4), a steroid hormone secreted from luteal steroidogenic cells, attenuated PRL-induced apoptosis in cultured luteal cells in a dose-dependent manner. P_4 significantly decreased the expression of mRNA of Fas, but not FasL, in cultured luteal cells prepared from both proestrous and mid-pseudopregnant rats. These data indicate that P_4 suppresses PRL-induced luteal cell apoptosis via reduction of the expression level of Fas mRNA in the corpus luteum, suggesting that P_4 acts as an important factor that can change the sensitivity of corpus luteum to PRL.

Key words: Apoptotic cell death; Fas; Fas ligand; Luteal regression; Progesterone

1. Introduction

Apoptosis, a form of programmed or physiological cell death, allows tissue remodeling and cellular replacement to occur rapidly without damaging surrounding tissue [1]. One of the physiological processes in which apoptosis takes place is the regression of the corpora lutea in the ovary [2]. In rats, when pregnancy fails to occur, the corpora lutea should be rapidly removed from the ovary for induction of the next ovulation. We have previously found that luteolysis (the regression of the corpus luteum) in rats is associated with the occurrence of fragmented DNA degeneration, a hallmark of apoptosis, and that this degeneration depended on the presence of endogenous surge levels of PRL [3,4].

Recent observations have revealed an involvement of the Fas/APO-1/CD95 (Fas) and Fas ligand (FasL) system with reproductive tissue remodeling [5–12]. Fas is a cell surface molecule that mediates apoptosis-inducing signals by stimulation with Fas ligand or agonistic anti-Fas antibody [13]. Large amounts of Fas protein and mRNA have been detected in mammalian reproductive as well as immune tissues [7–10,14,15]. Treatment with anti-Fas antibody has been shown to induce apoptosis in human and mice primary cultured cells prepared from corpora lutea [7,16] and to enhance both follicular atresia and structural luteolysis in vivo [8,9]. Our recent studies have demonstrated that PRL-induced luteal steroidogenic cell apoptosis also involves the Fas/FasL system in rats [17]. FasL expression is enhanced by PRL in cultured rat luteal immune cells, and PRL-induced luteal steroidogenic cell apoptosis is inhibited by the addition of anti-FasL antibody that neutralizes FasL function, indicating that the Fas/FasL system plays a crucial role in the luteolysis initiated by PRL.

Once pregnancy or pseudopregnancy is introduced in female rats, however, the corpus luteum does not undergo apoptosis against endogenous PRL surges until termination of the pregnancy/pseudopregnancy [18–23]. The corpus luteum contains many luteal steroidogenic cells with hyperplastic and hypertrophic features that continuously secrete progesterone (P_4) for the maintenance of pregnancy/pseudopregnancy. We have revealed that the addition of PRL to primary cultured luteal cells prepared from these pseudopregnant female rats fails to induce apoptosis [4]. Thus, luteal steroidogenic cells in pregnancy seem resistant against the apoptotic action of PRL, whereas these corpora lutea during the estrous cycle rapidly regressed and eventually disappeared as a result of apoptotic processes by PRL. Therefore, the issue is what determines the tissue and cell fate of corpus luteum in between the estrous cycle and pregnancy/pseudopregnancy, especially in terms of the response against the apoptotic function of PRL.

In the present study, we revealed that P_4 acts as an important factor that changes the sensitivity of the corpus luteum to PRL. P_4 suppresses PRL-induced luteal steroidogenic cell apoptosis via a reduction of the expression levels of Fas mRNA in the corpus luteum, suggesting the existence of an autonomous tissue/cell fate decision mechanism in the regression of corpus luteum.

2. Materials and methods

2.1. Animals

Adult female Wistar rats purchased from Imamichi Institute for Animal Reproduction (Omiya, Japan) (12 weeks old, 313.1 ± 5.8 g body weight (BW)) were housed under a controlled lighting condition of 14 h light and 10 h darkness for 1–2 months (lights on 05:00–19:00 h). All animals received human care as outlined in the 'Guide for the Care and Use of Animals' (Tokyo University Animal Care Committee according to NIH #86-23; revised 1985). Vaginal cytological examinations were carried out every day, and only those animals showing consecutive regular 4-day cycles were used. Luteal cells were obtained from 8- to 14-week-old rats on the proestrous day as previously described [17]. Pseudopregnancy was induced by cervical stimulation with a glass rod between 18:00–19:00 h on the proestrous day, and

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the next day (the day of estrus) was designated as day 1 of pseudopregnancy.

2.2. Cell preparation, culture, and assay for cell viability

Cells were prepared from corpora lutea by a modified method of Kuranaga et al. [17]. In our preparation method, 'luteal cells' contain both steroidogenic cells and immune cells. To examine the rate of steroidogenic cells in luteal cells prepared from rat corpora lutea, we used 3β-hydroxysteroid dehydrogenase (3β-HSD) activity staining, as described above [17]. The rate of steroidogenic cells was 39.2 ± 1.8% in luteal cells. For the cell viability assay, the number of viable cells was assessed using a Dojindo Cell Counting Kit including WST-1 (Dojindo), a kind of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium/Br), as previously described [17]. In this assay, data are expressed as the percentage of the appropriate control values.

2.3. Detection of fragmented DNA by gel electrophoresis

The metestrous and diestrous rats were given s.c. injections (15:00 h, 16:00 h and 17:00 h) with PRL (ovine PRL; Sigma; 667 μg/kg BW) or saline. A dose of PRL was used as determined in previous studies by Day et al. [22]. Approximately 30 corpora lutea were collected and weighed at 18:00 h on the metestrous and diestrous days. Fragmented DNA was isolated and analyzed by a previously reported method [3,24,25]. Densitometric analysis of low molecular weight DNA was performed with an Image Scanner (EU10, Epson Co., Tokyo, Japan) with NIH Image 1.57 software (Wayne Rasband, National Institute of Health, USA) as previously described [9]. The trials were repeated more than three times for each sample, and representative results are shown.

2.4. Detection of Fas and FasL mRNA by RT-PCR

Expression of Fas and FasL mRNA in the cultured luteal cells was confirmed using RT-PCR. The primer sets for rat Fas (5′-GTG ATG AAG GGC ATG GTT-3′ and 5′-TTG ACA CGC ACC AGT CTT-3′) and rat FasL (5′-CCA GAT CTA CTG GGT AGA-3′ and 5′-ATG GTC AGC AAC GGT AAG-3′) were used. The reaction mixtures were heated at 95°C for 10 min, followed by 35 cycles of amplification steps carried out at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The samples were then incubated at 72°C for 10 min. The expected sizes of the PCR products were 546 bp (rat Fas) and 698 bp (rat FasL). The equality of the amount of cDNA samples used for PCR of rat Fas and FasL was verified by PCR using the primer set of rat β-actin (Research Genetics, Inc) (PCR cycle: 25 cycles). Quantitative analysis of the expression of Fas, FasL and β-actin mRNA were performed on the photo of the gel using NIH image software.

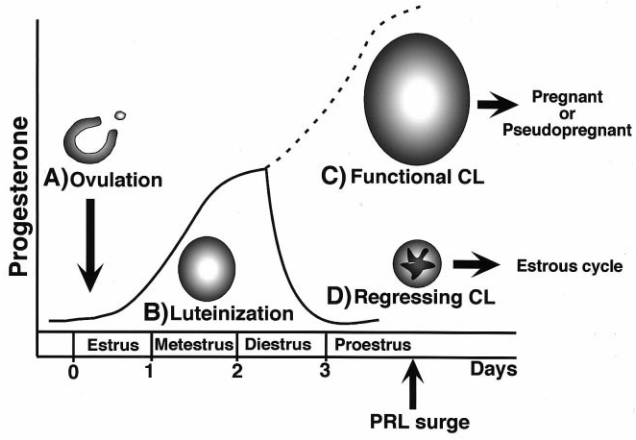
2.5. Statistical analysis

The Student's *t*-test was used for statistical evaluation of all the results. Differences of $P < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Luteal cells in each estrous cycle stage exhibit different response against PRL

In cycling female rat, the corpus luteum is formed on the day of estrus, and serum P_4 levels start increasing on the next day (metestrus), rapidly decrease upon the start of diestrus, and then remain low during proestrus (Scheme 1). When pregnancy/pseudopregnancy is achieved by mating, the corpus luteum becomes fully functional for P_4 secretion and contains many hypertrophic and hyperplastic luteal cells. These functional corpora lutea lose their sensitivity to PRL surges, which can cause luteolysis [4] (Scheme 1). We hypothesized that such a completely different response of corpus luteum against PRL might be modulated by some of the hormones that regulate the female estrous cycle like LH, FSH, estrogen, androgen, P_4, GnRH, and so on. To test this possibility, the luteal cells were prepared on the day of proestrus, metestrus, and diestrus, and we then examined whether the luteolytic action of



Scheme 1. A scheme for the fate of corpus luteum in relation to the serum concentrations of serum P_4 levels based on a report by Smith et al. [23] in the cycling rat (a solid line) and the pregnant rat (a dotted line). A: First, ovulation occurs at the estrous stage. B: Second, luteinization, the formation of the corpus luteum, is observed in the ovary after ovulation. C: In the case of a successful mating, the corpus luteum maintains P_4 secretion (functional corpus luteum). D: In a normal estrous cycle, the serum P_4 level is rapidly decreased during the diestrous afternoon, and corpus luteum begins to regress following the PRL surge at the proestrous stage.

PRL on the cells during proestrus could be reproduced in the cells at other stages of the estrous cycle. Consistent with previously reported data [3,17], the addition of PRL caused a significant decrease in the viability of proestrous luteal cells (proestrus 09:00, 12:00, and 18:00 h; Fig. 1). Because we were able to observe a similar apoptotic effect of PRL on luteal cells prepared from diestrous female rats (diestrus 18:00 h; Fig. 1), we concluded that a stage-specific endocrine milieu in vivo is not involved in this apoptotic process initiated by PRL. However, PRL could reproduce an apoptotic cell death process in luteal cells of diestrus (diestrus 18:00 h; Fig. 1), but not metestrus (metestrus 18:00 h; Fig. 1), suggesting that apoptosis inhibitory mechanisms against PRL-induced luteolysis may exist in the metestrous corpus luteum.

3.2. PRL can induce apoptosis in the corpus luteum in the diestrous stage, but not in metestrus in vivo

To examine these observations precisely, we intended to inject PRL into female rats and recover corpora lutea for evaluating the amounts of fragmented DNA. Injection of PRL on the day of diestrus significantly induced DNA fragmentation in the corpora lutea, but the corpora lutea on the metestrous day failed to undergo apoptosis in response to PRL treatment (Fig. 2). These data clearly indicated that the critical point at which the cell/tissue fate of corpus luteum divides between survival and regression should be in between metestrus (18:00 h) and diestrus (18:00 h). One of the hormonal events that occurs during this limited period is a decrease in serum P_4 levels (Scheme 1), and this leads us to hypothesize that P_4 may be involved in the regulations of corpus luteum regression, especially the execution of luteal cell apoptosis.

3.3. P_4 inhibits PRL-induced apoptosis in luteal cells

Our observations described above initially suggested that P_4 can function to suppress the occurrence of apoptosis induced by PRL in the corpus luteum. To examine this possibility, P_4

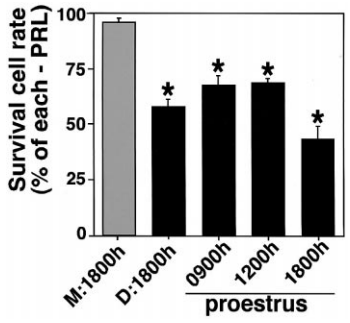


Fig. 1. Luteal cells in each estrous cycle stage exhibit different responses against PRL. Stage-dependent effects of PRL on the viability of cultured luteal cells prepared from corpora lutea at each stage (M: metestrus 18:00 h, D: diestrus 18:00 h, proestrus 09:00 h, 12:00 h, 18:00 h) in the estrous cycle. Luteal cells were cultured for 24 h in the presence of PRL (2 μg/ml). The cell viability was measured by MTT assay. The results are expressed as percentages of the average viability of the control cells cultured without PRL (mean ± S.E.M., $n = 4$, $^*P < 0.01$).

was added with PRL to primary luteal cells prepared from the proestrous (18:00 h) rat. As shown in Fig. 3, PRL-induced luteal cell apoptosis was significantly suppressed by the addition of P_4 in a dose-dependent manner. In this result, because PRL-induced luteal cell apoptosis was observed to be effectively suppressed by P_4, the ability of luteal cells to secrete P_4 would determine whether the corpus luteum survived or regressed. In female rats, functional regression of the corpus luteum with a decrease in serum P_4 concentrations begins within 2–3 days after ovulation by an acute increase in the activity of 20α-hydroxysteroid dehydrogenase (20α-HSD), which catabolizes P_4 into an inactive steroid, 20α-dihydroprogesterone [26]. Therefore, luteal cells expressing active 20α-HSD are likely to be functionally and fatally 'dead cells' that should be eliminated via apoptosis induced by the PRL surge that occurs on the day of proestrus.

3.4. P_4 downregulates Fas mRNA expression in proestrous luteal cells

We next tried to determine how P_4 suppresses PRL-induced luteal cell apoptosis. We previously observed that PRL stimulates the expression of FasL on luteal immune cells, and the Fas-expressing luteal steroidogenic cells are induced to apoptosis by this FasL ([17] and our unpublished results). Thus, based on these results, we hypothesized that P_4 may affect the expression levels of Fas or FasL in the cells of the corpus luteum. To elucidate this hypothesis, semi-quantitative RT-PCR for the detection of Fas and FasL mRNA was performed on cultured luteal cells. In primary luteal cell culture prepared from a proestrous (18:00 h) female rat, both Fas and FasL mRNA were expressed abundantly at a 0 h culture period (Fig. 4A). When luteal cells were incubated with P_4 (2 μg/ml) for 24 h, the Fas expression, but not FasL, was dramatically reduced by the addition of P_4 (Fig. 4A), indicating that P_4 has an ability to downregulate Fas mRNA expression in the cells of the corpus luteum. In each experiment, there was no observable change in β-actin mRNA levels (Fig. 4A and B).

3.5. Fas mRNA expression is increased by the withdrawal of P_4 in pseudopregnant luteal cells

We next prepared primary luteal cells from the mid-pseudopregnant female rat, in which serum concentrations of P_4 were maintained at high levels because 20α-HSD activity in the corpus luteum is significantly suppressed [18–22]. At a 0 h culture period, the levels of Fas and FasL mRNA expression were revealed to be low in pseudopregnant luteal cells (Fig. 4B). The amount of Fas mRNA was remarkably increased when these luteal cells were cultured without P_4 for 24 h and 48 h, suggesting that Fas expression in corpus luteum may be suppressed by high concentrations of serum P_4 in the mid-pseudopregnant female rat. Actually, the Fas mRNA expression remained at low levels with the continuous addition of P_4 into the culture medium. Thus, these observations suggest that P_4 suppresses the PRL-induced luteal steroidogenic cell apoptosis by the downregulation of Fas expression.

Based on these results, an outline of a predicted mechanism for luteolysis, specifically a regression of the corpus luteum, can be described as follows: first, the steroidogenic cells in the

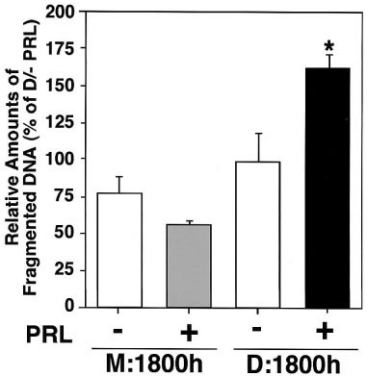


Fig. 2. PRL induces apoptosis in the corpus luteum at the diestrous, but not the metestrous, stage in vivo. The donor rats were pretreated three times by injection (15:00 h, 16:00 h, and 17:00 h) of either PRL (667 μg/kg BW) or saline alone as control. Samples were collected each day at 18:00 h, and a performed densitometric analysis of the electrophoresed DNA fragments was performed. The relative amount of fragmented DNA at diestrus 18:00 h (PRL−) was taken as 100%. (mean ± S.E.M., $n = 4$, $^*P < 0.05$).

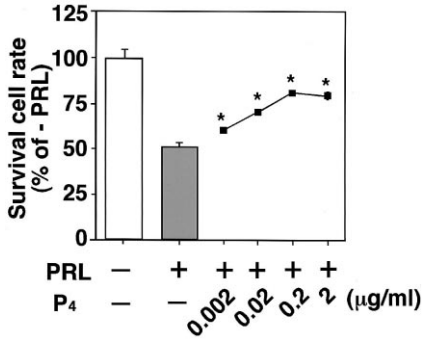


Fig. 3. P_4 inhibits PRL-induced apoptosis in luteal cells. A dose-dependent effect of P_4 was observed on the viability of cultured luteal cells prepared from corpora lutea at proestrus 18:00 h. Luteal cells were cultured for 24 h in the presence of graded concentrations of P_4. After this treatment, the culture medium was changed, and luteal cells were cultured with PRL (2 μg/ml) in the presence of each concentration of P_4. The cell viability was measured by MTT assay. The results are expressed as percentages of the average viability in control cells cultured with the vehicle (mean ± S.E.M., $n = 8$, $^*P < 0.05$ vs. +PRL/−P_4).

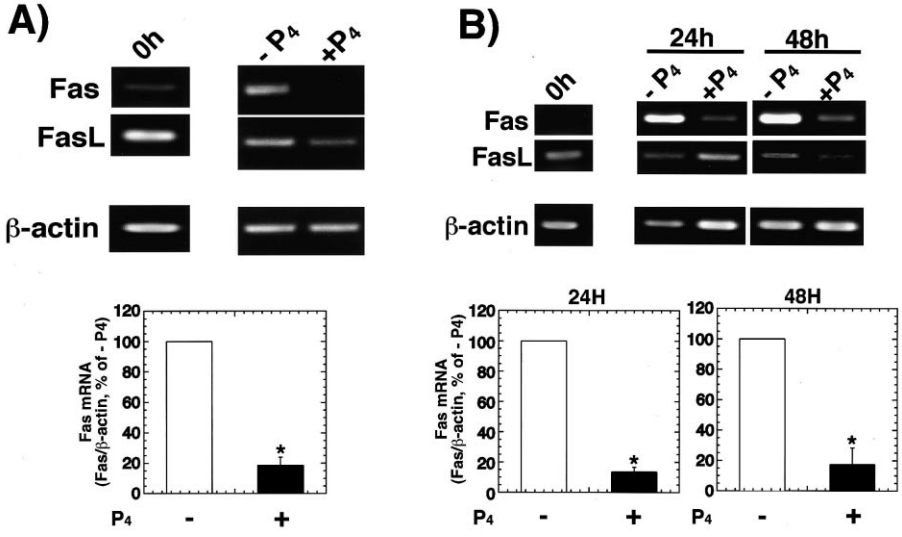


Fig. 4. P_4 downregulates Fas mRNA expression in luteal cells. Semi-quantitative RT-PCR analysis of the expression of rat Fas and FasL mRNA in the cultured luteal cells. A: Expressions of Fas and FasL mRNA in cultured luteal cells from female rats at the day of proestrus 18:00 h. Luteal cells were cultured for 0 h (left panel), and 24 h (right panel) in the presence of P_4 (2 μg/ml). Control cells were cultured with the vehicle. RT-PCR was performed by using specific primers for the detection of rat Fas and FasL. These signals were normalized to the β-actin internal control for each sample (lower panel). The quantification data (the ratio of Fas and FasL to each β-actin PCR produces) are expressed as the ratio to the average amount in samples taken at $-P_4$. Data points represent the mean ± S.E.M. of four separate experiments ($^*P < 0.01$ vs. $-P_4$). B: Expressions of Fas and FasL mRNA in cultured luteal cells from mid-pseudopregnant rats (day 6). Luteal cells were cultured for 0 h (left panel), 24 h (middle panel), and 48 h (right panel) in the presence of P_4, as described in A.

corpus luteum, which exhibit 20α-HSD activity and lose their P_4-secreting ability, begin to express Fas on their surface; second, PRL binds to its receptor on luteal immune cells and induces the expression of FasL on the surface of these kind of cells ([17] and our unpublished results); and third, FasL-expressing immune cells interact with Fas-expressing steroidogenic cells and induce apoptotic cell death in these steroidogenic cells [17].

Thus, in the mechanism of rat luteal regression, the level of P_4 concentration determines the fate of corpus luteum by downregulating Fas expression, suggesting the novel cell-autonomous regulation of physiological cell death.

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