Requirement of the Fas ligand-expressing luteal immune cells for regression of corpus luteum

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Received 24 December 1999; received in revised form 6 March 2000

Edited by Masayuki Miyasaka

Abstract Apoptosis in corpus luteum (CL) is induced by prolactin (PRL) in female rats. PRL-induced apoptosis in CL is mediated by the Fas/Fas ligand (FasL) system. The CL consists of steroidogenic and non-steroidogenic cells, including immunocytes. Fas mRNA was detected only in the luteal steroidogenic cells, and FasL mRNA was expressed only by the non-steroidogenic CD3-positive luteal immunocytes. Removing the luteal immune cells from the luteal cells inhibited PRL-induced luteal cell apoptosis effectively. Thus, FasL-expressing non-steroidogenic luteal immunocytes are required for PRL-induced luteal cell apoptosis and heterogeneous induction of apoptosis by Fas/FasL in CL.

Key words: Apoptosis; Cell death; Fas/Fas ligand; Rat corpus luteum; Luteolysis; Luteal immune cell

1. Introduction

The regression of corpus luteum (CL) in female rats (luteolysis), assessed by decreases in weight, size and steroidogenic activity, has been shown to be dependent on the prolactin (PRL) surge that occurs in the proestrous afternoon [1–4]. We previously found that this luteolysis was associated with the occurrence of fragmented DNA degeneration, a hallmark of apoptosis [5,6]. This apoptotic regression of CL seems to be a suitable model for elucidating the processes of physiological cell death.

The CL tissue consists of steroidogenic and non-steroidogenic cells [7–18]. During luteinization, the steroidogenic follicular granulosa and theca cells differentiate to luteal steroidogenic cells, and simultaneously endothelial and blood cells migrate extensively into the newly formed CL tissue [13–17]. Progesterone is synthesized in and secreted from steroidogenic luteal cells; however, the maintenance of this function has been believed to be supported by other cell populations such as fibroblast, endothelial, and blood cells [11,13–17,19,20].

Previous reports suggest the involvement of immune cells during the process of PRL-induced luteolysis; Bowen et al. [21] reported the invasion of macrophages and an increased expression of monocyte chemo-attractant protein-1 (MCP-1) during PRL-induced CL regression, and PRL-induced luteolysis was blocked by immunosuppressive levels of glucocorticoid [22] and a prostaglandin synthesis inhibitor [23]. Furthermore, recent observations revealed the correlation of the Fas/Fas ligand (FasL) system with reproductive tissue remodeling [24–28]. Fas is a cell surface molecule that mediates apoptosis-inducing signals by stimulation with FasL or agonistic anti-Fas antibody [29]. A significant amount of Fas is detected in mammalian reproductive organs as well as in immune tissues [24–27,30]. Our recent studies have demonstrated that PRL-induced apoptosis at luteolysis is mediated by the Fas/FasL system in rats [31]. Briefly, FasL expression was enhanced by PRL stimulation on cultured rat luteal cells, and PRL-induced luteal cell death was inhibited by the addition of neutralizing anti-FasL antibody [31]. We have also suggested the possible roles of luteal immune cells in PRL-induced luteolysis, by the observations that FasL expression in luteal cells was enhanced by concanavalin A (ConA), a reagent for T-cell stimulation, and that both CD3-positive and FasL-positive cells were co-localized in regressing CL in discrete regions where apoptosis occurred [31].

In this study, we defined specific cell populations that express FasL in CL and found that FasL and Fas receptor-expressing cell populations in CL are dissociated. The FasL-expressing cells are non-steroidogenic cells including CD3-positive immune cells, which are therefore essential for PRL-induced luteolysis. Our observations present a novel physiological cell death involving FasL-expressing killer cells, which results in such rapid tissue remodeling as regression of CL.

2. Materials and methods

2.1. Animals

Adult female Wistar rats (12 weeks old) were housed under controlled lighting conditions of 14 h light and 10 h darkness for 1–2 months (lights on 05.00–19.00 h). Vaginal cytological examination was done every day, and only those animals that had shown consecutive regular 4-day cycles were used. Luteal cells were obtained from 8–14-week-old rats on the proestrous day as previously described [31].

2.2. Dispersion, separation and culture of luteal cells

Cells were prepared from CL by a modified method of Kuranaga et al. [31]. Briefly, a crop of newly formed CL were removed from 8–14-week-old rats at 18.00 h on the proestrous day and dispersed by collagenase.

To separate luteal cells into populations based on size and gravity, a modified Percoll gradient centrifugation (Pharmacia) method was used according to the procedure of Saksela et al. [32]. Briefly, dispersed cells in 3 ml Dulbecco's modified Eagle's medium (DMEM) were quietly put on 3 ml 10% Percoll solution. The column was centrifuged at 500 rpm at 4°C for 7–9 min, until the large cell subpopulation of prepared cells moved into the Percoll solution. Fraction 1 was 2 ml of the top layer, and fraction 2 was 2 ml of the lower layer. Each fraction was collected in a 15-ml tube and centrifuged at 1000 rpm at 4°C for 10 min.

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PII: S0014-5793(00)01426-5

The cells were seeded in 96-well plates at a density of 32 000 viable cells/0.25 ml/well and cultured in a medium consisting of DMEM supplemented with 10% fetal bovine serum, 20 mM HEPES, 50 IU/ml penicillin, and 0.1 mg/ml streptomycin.

2.3. Detection of steroidogenic cells

Steroidogenic cells in cultured luteal cells were identified by both 3β-hydroxysteroid dehydrogenase (3β-HSD) and 20α-HSD activity stainings. Each of the two cell populations was washed in phosphate-buffered saline (PBS) and incubated with reactive solution in PBS containing 1.16 mM nicotinamide, 0.18 mM nitroblue tetrazolium, 0.72 mM β-NADP, 6.25% propylene glycol, 1.25 mM EDTA, 86.7 μM dehydroepiandrosterone (for 3β-HSD activity staining) or 10 mg/ml 20α-dihydroprogesterone (for 20α-HSD activity staining), and 0.1% bovine serum albumin for 60 min at 37°C. The frequency of steroidogenic cells was calculated by counting the number of blue-stained cells in the total number of cells.

2.4. Western blotting

To characterize cell types in the large and small cell populations, Western blotting was done with monoclonal anti-rat CD3 antibody, a specific antibody to rat T-lymphocytes, as previously reported by Kuranaga et al. [31]. Samples were electrophoresed on a 15% polyacrylamide gel, and the protein was transferred to PVDF membranes (Millipore). The membranes were incubated for 1 h at room temperature with 1000-fold diluted anti-rat CD3 antibody (Cerotec), and then incubated for 30 min at room temperature with 1000-fold diluted peroxidase-conjugated rabbit anti-mouse IgG (A0207; Vector Laboratories Inc.). The proteins recognized by the antibody were visualized by the ECL system (Amersham).

2.5. Immunostaining

Small cells treated with Percoll were used for immunostaining. Cells were immersed in 4% PFA in PBS and preincubated with 4% skim milk in PBS. Then, cells were incubated with rabbit anti-rat FasL antibody (C-178; Santa Cruz Biotechnology, Ltd.) (1:100) and anti-CD3 monoclonal antibody (1F4; Cerotec) (1:100) diluted in skim milk for 90 min at room temperature. Thereafter, small cells were incubated with TRITC-conjugated swine anti-rabbit Ig (R-0156; Dako) (1:100) and FITC-conjugated goat anti-mouse Ig (F-0479; Dako) (1:100) for 60 min at room temperature.

2.6. Detection of Fas, FasL, 20α-HSD and PRL receptor (PRL-R) mRNAs by RT-PCR

Expression of Fas, FasL, 20α-HSD and PRL-R mRNAs in the cultured luteal cells was analyzed using RT-PCR. The TRIzol reagent (Gibco BRL) was used to extract total RNA from cultured luteal cells. cDNA was synthesized from 500 ng of total RNA using oligo-d(T) (Perkin-Elmer) and Ready-to-Go You-Prime First-Strand Beads (Promega, Annandale, NSW, Australia). For PCR analysis, the primer sets for rat Fas (5′-GTG ATG AAG GGC ATG GTT-3′ and 5′-TTG ACA CGC ACC AGT CTT-3′), rat FasL (5′-CCA GAT CTA CTG GGT AGA-3′ and 5′-ATG GTC AGC AAC GGT AAG-3′), rat 20α-HSD (5′-CTA GGG AAG AGC AGC ATC TGA-3′ and 5′-AGC AAG ACC AAC GGA TAC CAC-3′) and rat PRL-R (5′-TCT CCA GCA GAT GGG TAT CAA AT-3′ and 5′-GAG TGG GAG ATC CAT TTT ACA GG-3′) were used. Reaction mixtures were heated at 95°C for 10 min, followed by 35 cycles of amplification steps comprising 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Then the samples were incubated at 72°C for 10 min. The expected sizes of each PCR product were 546 bp (rat Fas), 698 bp (rat FasL), 675 bp (rat 20α-HSD), and 632 bp (rat PRL-R). The equality of the amount of cDNA samples used for PCR was verified by PCR using the primer set for rat β-actin (Research Genetics, Inc). The trials were repeated more than three times for each sample, and representative results are shown.

2.7. Assay for cell viability

For cell viability assay, the amount of viable cells was assessed using a Dojindo Cell Counting Kit including WST-1 (Dojindo) as

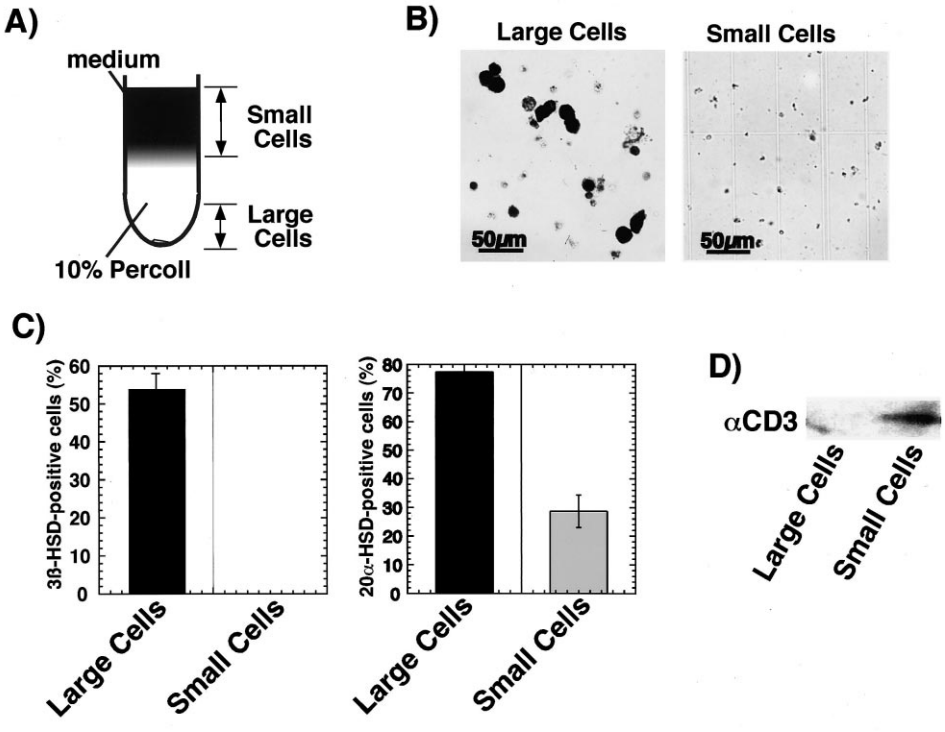

Fig. 1. Characterization of Percoll-treated rat luteal cells. A: Scheme of the Percoll-treated method. Dispersed luteal cells in 3 ml DMEM were quietly put on 3 ml 10% Percoll solution, and the column was centrifuged until the large cell subpopulation in the prepared cells moved into the Percoll solution. Fraction 1, 2 ml of the top layer (small cell fraction); fraction 2, 2 ml of the lower layer (large cell fraction). B: 3β-HSD activity staining of small (right panel) and large (left panel) luteal cells after separation by Percoll treatment. C: The steroidogenic cells were identified by 3β-HSD and 20α-HSD activity staining (mean ± S.E.M., $n = 4$, $^*P < 0.01$ vs. large cells). D: Western blot analysis of CD3 in each fraction. Cell lysates from each cell population were analyzed by immunoblotting with anti-CD3 antibody. CD3 protein was expressed in the small cells but not in the large cells.

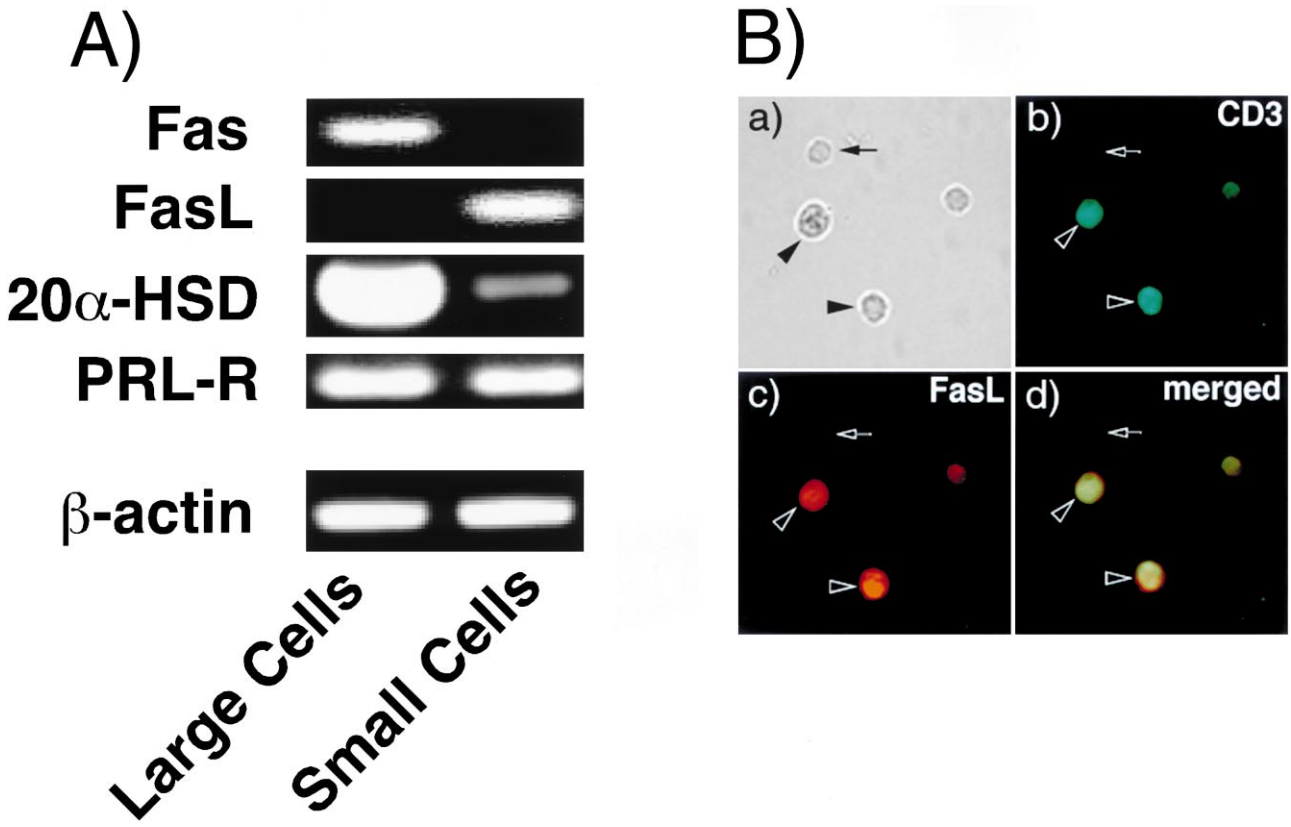

Fig. 2. Characterization of Fas and FasL contained in each cell fraction. A: Expressions of Fas, FasL, 20α-HSD and PRL receptor mRNA in large and small cell fractions were confirmed using RT-PCR. Fas mRNA was detected only in the large cell fraction, and FasL mRNA was detected only in the small cell fraction. 20α-HSD and PRL receptor were expressed in both large and small cell fractions. To reveal that each sample nearly included equivalent mRNA, we measured the expression of β-actin mRNA. B: Immunostaining of the small cell fraction of luteal cells. a: light phase, b: CD3 (green), c: FasL (red), d: merged (yellow). Arrowheads indicate the cells that express both CD3 and FasL. A non-stained cell was observed in all panels (arrow).

previously described [31]. In this assay, data are expressed as percentages of the appropriate control values.

2.8. Statistical analysis

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

3. Results

3.1. Characterization of the large and small cell fractions

To determine the cell that expresses Fas and FasL in CL, we first intended to separate and characterize the subpopulations of CL cells. As shown in Fig. 1A,B, dispersed luteal cells were successfully dissociated according to cell size. To assess which kind of cells were included in each cell fraction, first the steroidogenic cells were identified by 3β-HSD and 20α-HSD activity stainings. The large cell fraction consists mainly of 3β-HSD- and 20α-HSD-positive cells ($53.92 \pm 4.03\%$ and $77.36 \pm 6.12\%$, respectively), but in the small cell fraction, these steroidogenic activities were exhibited only by a minor part of the cells (3β-HSD; $0.01 \pm 0.00\%$, 20α-HSD; $28.58 \pm 5.62\%$, Fig. 1B,C). The mRNA of 20α-HSD was also mainly detected in the large cell fraction (Fig. 2A).

Next, these two cell fractions were subjected to Western blotting with anti-CD3 antibody for detecting immune cells, especially peripheral T-cells, in the luteal tissue. The CD3 proteins were observed in the small cell fraction, but not in the large cell fraction (Fig. 1D). These data suggest that most steroidogenic cells in CL are separated into the large fraction, and most of the luteal immunocytes, including CD3-positive T-cells, are separated into the small cell fraction.

3.2. Differential expression patterns of Fas and FasL mRNA in the luteal cell populations

To define the expression of Fas and FasL mRNAs in either the small or the large cell fraction, semi-quantitative RT-PCR was performed, together with the expression of PRL receptor (PRL-R) and progesterone receptor mRNAs, which are known to be expressed in CL. As shown in Fig. 2A, abundant Fas mRNA was expressed specifically in the large cell fraction, which was consistent with our previous result that luteal cell apoptosis induced by PRL mainly occurs in steroidogenic cells [31]. The fractionated small cell population, however, expressed only FasL but not Fas mRNA (Fig. 2A), suggesting that the small cell fraction includes killer-type cells that induce cell death against Fas-expressing steroidogenic cells by FasL. PRL receptor mRNA was detected in both large and small cell fractions (Fig. 2A). Thus, FasL-expressing cells are different from Fas-expressing cells in regressing CL; the former may be killer-type immune cells and the latter may be steroidogenic cells.

In addition, immunohistochemical analysis was performed to identify FasL-expressing cells in the small cell fraction. Immunohistochemical analysis revealed that most CD3-positive cells also expressed FasL (Fig. 2B). We also stained the

small cell fraction by ED1, a specific marker for macrophages. However, macrophage-like cells could not be detected in the small cell fraction (data not shown). Thus, we suggest that FasL is expressed in the CD3-positive luteal immune cells.

3.3. Small non-steroidogenic cells are required for PRL-induced luteal cell apoptosis

To elucidate whether the small cell fraction containing FasL-expressing cells is necessary for the luteal cell apoptosis induced by PRL, the fractionated large cells were cultured with or without the small cell fraction. Although a considerable decrease in cell viability was observed even in the absence of PRL, the data in Fig. 3 are expressed as percentages of the average viability in the absence of PRL to evaluate the PRL effect in vitro. The large steroidogenic cells co-cultured with small cells were rendered apoptotic by the addition of PRL (Fig. 3A) exactly as was observed in a whole cell culture [31,42]. However, PRL induced apoptosis in neither the fractionated large cell culture nor the fractionated small cell culture (Fig. 3B,C). We confirmed that the large steroidogenic cells did not express FasL mRNA by PRL addition after the culture period (data not shown). These data strongly indicate that the FasL-expressing small cells containing luteal immunocytes are required for PRL to induce apoptosis in steroidogenic cells.

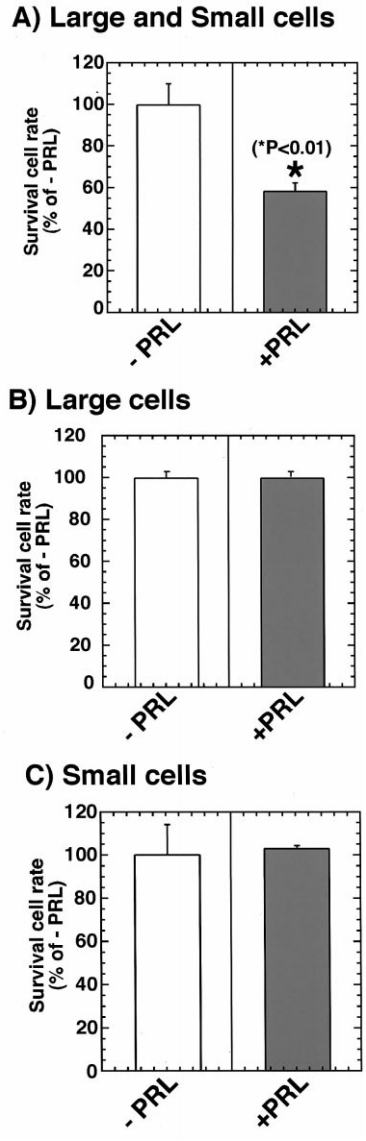

Fig. 3. Small cell fraction is required for PRL-induced luteal cell apoptosis. Large fractionated cells were cultured with or without the small cell fraction in the presence of PRL (2 μg/ml). Luteal cells were cultured for 24 h. Cell viability was measured by MTT assay. The results are expressed as percentages of the average viability in the absence of PRL (mean ± S.E.M., $n = 4$ $^*P < 0.01$ vs. control). A: Large steroidogenic cells were co-cultured with small cells, and were induced to apoptosis by PRL stimulation. Single cultured large fractionated cells (B) and small fractionated cells (C) were not induced to apoptosis by PRL stimulation.

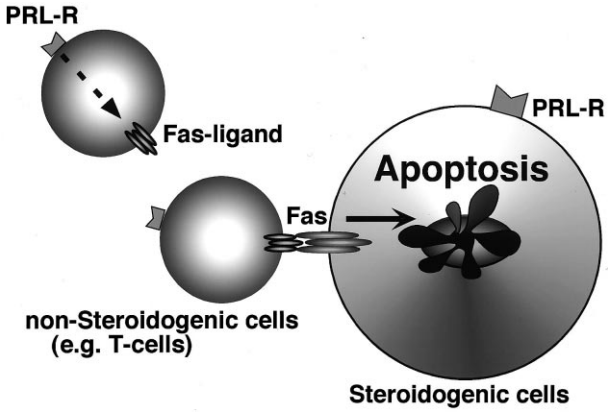

Fig. 4. A model for the role of luteal large (steroidogenic) and small (non-steroidogenic) cells in luteal cell apoptosis and the outline of a predicted mechanism for CL regression. The FasL expression is enhanced by PRL stimulation on the non-steroidogenic cells. Then, the non-steroidogenic cells (mainly immunocytes) expressing FasL interact with Fas-expressing steroidogenic cells and induce apoptotic cell death in steroidogenic cells.

4. Discussion

The present study shows the first clear demonstration of a physiological role for luteal non-steroidogenic cells, which are required for inducing apoptosis in luteal steroidogenic cells. The CL is a heterogeneous tissue consisting of steroidogenic and non-steroidogenic cells [12–18]. The separation of luteal cells has been done for different intentions to obtain large and small steroidogenic cell populations [18,33–36]. In this work, however, our intention was to separate non-steroidogenic cells from the steroidogenic luteal cell population, and we succeeded in separating the non-steroidogenic cell population into the 'small cell fraction'; most of the steroidogenic cell population was segregated into the 'large cell fraction'. It is noteworthy that expression of CD3 (a marker for peripheral T-cells) was only detected in the small cell fraction, suggesting that the small cell fraction contained immunocytes. Thus, we considered that these two subpopulations are useful for evaluating the exact roles of luteal immune cells in the process of apoptotic cell death in steroidogenic cells during luteolysis.

In cycling rats in which ovulation recurs at 4–5-day intervals, CL tissue never acquires the activity for maintaining continuous progesterone secretion [37–39]. This process has been described as structural luteolysis, and we recently revealed that structural luteolysis is initiated by apoptosis in luteal steroidogenic cells induced by PRL [31]. Because the

Fas/FasL system is required for this PRL-induced luteal cell apoptosis as we previously reported [31], luteal steroidogenic cells seem to be one of the candidates that express Fas to receive a cell death trigger, FasL. The present results indicate that CL cells in the way of regression can be classified into two cell populations according to their expressing molecules, Fas and FasL. The cells expressing Fas mRNA were included in the steroidogenic cell-rich population, and those expressing FasL mRNA were included in the non-steroidogenic cell population. In addition, immunohistochemical suggests that CD3-positive luteal immunocytes express FasL. Thus, although the possibility that fractionated small cells other than immunocytes also express FasL cannot be ruled out, the presence of non-steroidogenic luteal immune cells expressing FasL seems a prerequisite to induce apoptosis in Fas-expressing steroidogenic cells.

Although we previously showed the functional correlation of the Fas/FasL system with PRL-induced CL regression, it had remained to be elucidated whether or not the effector cells (with FasL) are identical to the recipient cells (with Fas): heterogenic or homogenic induction of apoptosis in CL regression. Because the addition of ConA as well as PRL enhances the expression of FasL protein in primary luteal cell culture [31], and because the morphological Fas expression pattern is clearly associated with the CD3-positive region in regressing CL [31], the cells that respond to PRL and express FasL have been suggested to be luteal immunocytes. In the present study, the following were found: (1) FasL-positive cells were specifically included in the small cell fraction where a majority of the cells were identified as non-steroidogenic cells; (2) CD3-positive cells in the small cell fraction express FasL proteins; and (3) the small cell fraction did not express Fas entirely. Thus, we conclude that during the course of structural luteolysis, the steroidogenic cells are killed by FasL-expressing luteal immune cells, but not by themselves. This style of apoptosis could be considered a non-cell autonomous form that resembles the immune response with the Fas/FasL system by cytotoxic T-cells.

By cell separation, we revealed that luteal non-steroidogenic cells are required for PRL-induced CL regression. Although some previous reports suggest that luteal immune cells may be required for the PRL-induced regression of CL by the use of immunosuppressive reagents [22,40,41], there remained other possibilities in causing damage to other tissues/cells or cell toxicity by these reagents, resulting in the artificial blockade of PRL-induced CL regression. Here we clearly showed that PRL-induced luteal cell apoptosis was inhibited in luteal cell culture without immune cells. Thus, it is directly suggested that the occurrence of luteal cell apoptosis requires the functions of luteal immune cells. This result also reinforces the notion that luteal cell apoptosis should be mediated by the Fas/FasL system. Based on these findings and previous observations [31,42], we can illustrate the predicted form of luteal cell apoptosis as follows (Fig. 4): in the regressing CL, (1) the steroidogenic cells express Fas on the surface on the cells' own surface, (2) PRL binds its receptor on luteal immune cells and induces the expression of FasL on the surface of this kind of cell, and (3) the cells expressing FasL interact with Fas-expressing steroidogenic cells and induce apoptotic cell death in steroidogenic cells rapidly.

In summary, we suggest that the FasL-expressing luteal immune cells are required for PRL-induced luteal cell apoptosis in the regressing CL. The Fas/FasL system in CL operates between the steroidogenic cells and the immune cells. These findings pose a novel fashion of physiological cell death using the Fas/FasL system in the remodeling of peripheral tissues, and can be implicated in other, unknown regulations of physiological apoptosis.

Acknowledgements: We are grateful to Dr. Keitaro Yamanouchi (Laboratory of Applied Genetics, Animal Resource Science, The University of Tokyo) for critical reading. This study was supported by 'Research for the Future' program of The Japan Society for the Promotion of Science (97L00904). H.K. is a research fellow of the Japan Society for the Promotion of Science.

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