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Fas/Fas Ligand System in Prolactin-Induced Apoptosis in Rat Corpus Luteum: Possible Role of Luteal Immune Cells

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The prolactin (PRL) surge in cycling rats during the proestrous afternoon is an inducer of apoptotic cell death in luteal cells. This luteolytic action of PRL is peculiar, because PRL may be categorized as a survival factor, if other known physiological functions of PRL are taken into account. Here we analyzed the underlying molecular/cellular mechanisms of this PRL-induced apoptosis. Corpora lutea (CL) were prepared from the ovary on the proestrous day and cultured with or without PRL (2 μg/ml). An addition of PRL to the culture medium induced DNA breakdown in the nuclei of cells mostly identified as steroidogenic by 3β-HSD activity staining, and the number of 3β-HSD-positive cells were significantly decreased, indicating the induction of apoptotic cell death by PRL among luteal cells in culture. Next, the expression of membrane form-Fas ligand (mFasL) in the luteal cell lysate was quantified, because Fas receptor is known to have an exact physiological role in luteolysis. An addition of PRL increased the expression of mFasL. Immunostaining and TUNEL assay on regressing CL revealed that both CD3-positive cells and FasL-positive cells were co-localized in the regions where apoptosis convergently occurred. Moreover, an addition of concanavalin A (ConA), a T-cell specific activator, to the culture mimicked the PRL action by inducing apoptosis in luteal cells and enhancing the expression of mFasL. These data suggest that the CD3-positive T lymphocyte in the CL is at least one of the PRL-effector cell species during the process of luteolysis in rats, and that FasL expression of these cells is upregulated by PRL.

The formation of corpora lutea (CL) is always followed by structural luteolysis and the occurrence of structural luteolysis is a prerequisite for maintaining normal cyclicity (1). The onset of structural luteolysis in the CL of cycling rats, assessed by decreases in weight, size and steroidogenic activity of the CL, has been shown to be dependent on the prolactin (PRL) surge that occurs in the proestrous afternoon (2–5).

We previously found that luteolysis in rats was associated with the occurrence of fragmented DNA degeneration, a hallmark of apoptosis, and that it depended on the presence of surge levels of PRL (9, 10). We also reported that PRL enhanced the cell viability of luteal cells prepared from functional CL of pseudopregnant rats (10). Our apparently contradictory findings give rise to the question of how PRL can act not only in a luteotropic fashion but also in a luteolytic fashion.

There are some reports suggesting an involvement of immune cells during the process of PRL-induced luteolysis. Bowen *et al.* (6) reported the invasion of macrophages and the antecedent enhancement of monocyte chemoattractant protein-1 (MCP-1) expression during PRL-induced luteolysis. This PRL-induced luteolysis was reported to be blocked by immunosuppressive levels of glucocorticoid (7) and a prostaglandin synthesis inhibitor (8). Moreover, recent observations have correlated the Fas/Fas ligand system with reproductive tissue remodeling (11–15). Fas is a cell surface molecule that mediates apoptosis-inducing signals by stimulation with Fas ligand (FasL) or agonistic anti-Fas antibody (16). It was reported that significant amount of Fas was detected in mammalian reproductive organ as well as in its immune tissues (11–14, 17). Further, anti-Fas antibody was shown to express apoptotic activity in human and mice luteal cells *in vitro* (11, 18)

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and to enhance either follicular atresia or structural luteolysis *in vivo* (12, 13).

To address the question of why PRL, which is classified as a typical growth factor and thus a survival factor, could lead to apoptotic cell death in regressing luteal cells, we attempted to identify the cell type expressing FasL in CL and to correlate the expression of this death-inducible molecule with the luteolytic action of PRL.

MATERIALS AND METHODS

Animals. Adult female Wistar rats (2 months old) were housed under a controlled lighting condition of 14 h light and 10 h darkness for 1-2 months (lights on 0500-1900 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- to 14-week-old rats on the proestrous day. The donors were pretreated with CB154, a dopamine agonist, so they would be exempted from the endogenous PRL surge. On the day of proestrus, between 1000 h and 1200 h, rats were given an s.c. injection of 2 mg/ml 2-bromo-α-ergocryptine (CB154, B-2134; Sigma) in 0.3% tartaric acid or the vehicle alone. A dose of 100 μl/300 g BW was used for the vehicle, as determined in previous studies by Day *et al.* (19). This treatment suppressed the endogenous PRL surge (9).

Ovaries for TUNEL assay and immunohistochemical evaluation of CD3 and Fas ligand antigens in the luteal tissue were obtained from intact cycling rats at the proestrous stage.

Cell preparation and culture. Cells were prepared from corpora lutea by a method descrived by Kanuka *et al.* (10). A crop of newly formed corpora lutea were removed from 8- to 14-week-old rats at 1800 h on the proestrous day and dispersed at 37°C with 0.4% collagenase (type 2; Worthington Biochemical) in HEPES buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 25 mM HEPES, pH 7.3) containing 0.4% BSA, 10 μg/ml DNase I, and 0.2% glucose. After two 25-min dissociation periods, the cells were washed twice and counted. The cells were viable in 91.1 $\pm$ 6.0%, as determined by trypan blue exclusion. To determine cell viability, the cells were seeded in 96-well plates at a density of 32,000 viable cells/0.25 ml/well and cultured in medium, consisting of DMEM supplemented with 10% fetal bovine serum, 20 mM HEPES, 50 U/ml penicillin, and 0.1 mg/ml streptomycin. Culture media were recovered for steroid hormone assay and changed every 24 h. Ovine PRL (Sigma) was added to the culture medium every day. The dose range of PRL was chosen according to our previous report (20).

Assay for cell viability. WST-1 (Dojindo), a kind of MTT (3-(4, 5-dimethyl-2 thiazolyl)-2,5-diphenyl-2H-tetrazolium/Br), is a yellow tetrazolium salt that is reduced to formazan by live cells containing active mitochondria. The viability of the cells was assessed using a Dojindo Cell Counting Kit including WST-1 (Dojindo). The culturing medium was aspirated and replaced with 100 μl medium A omitting phenol red, and a 10 μl solution (0.01% WST-1, 0.2 mM 1-methoxy PMS in 20 mM HEPES, pH 7.4) was added to each well. The cells were incubated for 4 h at 37°C, and absorbance (OD) was determined at 405 nm with a reference wavelength of 600 nm. The MTT assay was validated with freshly prepared rat luteal cells; OD increased linearly from 6,000 to 30,000 in viable cells. In this assay, data were expressed as percentages of the appropriate control values.

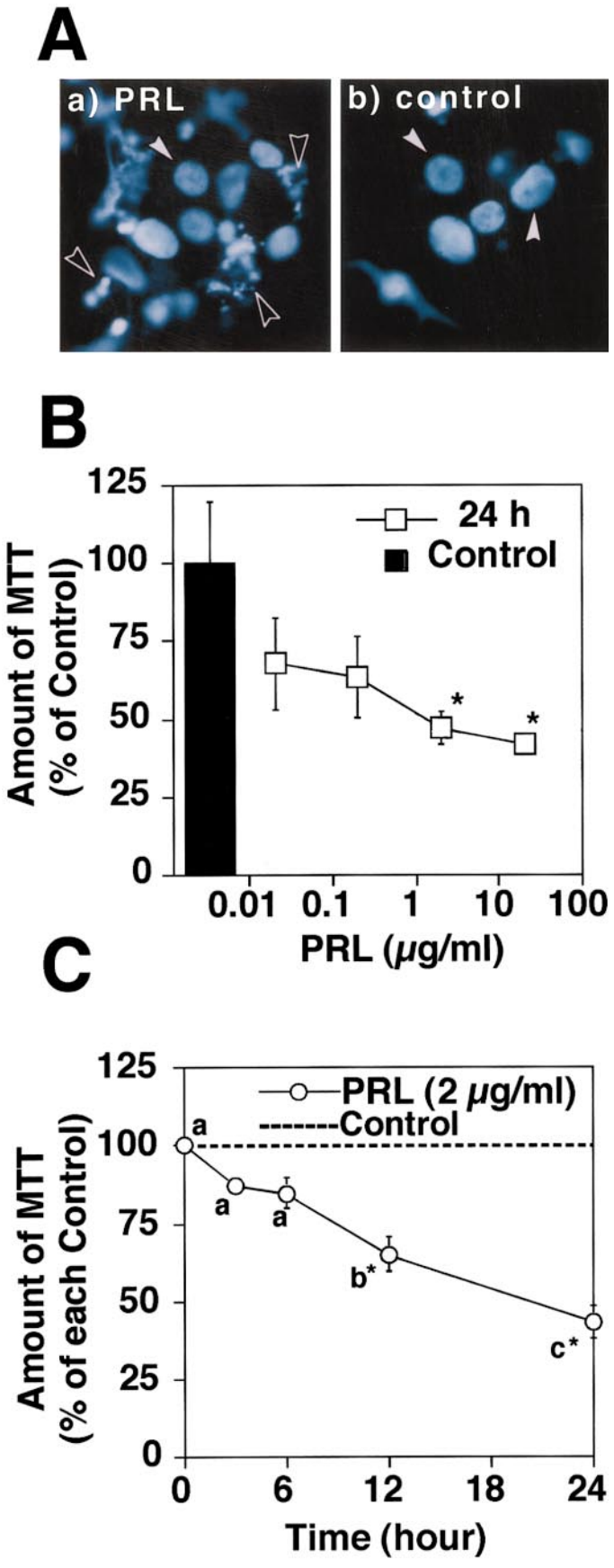
Detection of steroidogenic cells. Steroidogenic cells in cultured luteal cells were detected using 3β-hydroxysteroid dehydrogenase (3β-HSD) activity staining. Luteal cells were plated in 8-chamber slides at a density of approximately 1.25×10^5 cells/chamber in 0.5 ml medium A and cultured for 24 h. After the culturing period, these cells were washed in PBS and incubated with reactive solution in PBS containing 1.16 mM nicotinamide, 0.18 mM NBT, 0.72 mM βNADP, 6.25% propylene glycol, 1.25 mM EDTA, 86.7 μM dehydroepiandrosterone, and 0.1% BSA for 10 min at RT (room temperature). The frequency of steroidogenic cells was calculated by counting the number of blue-stained cells in the cultured cells.

Steroid hormone assay. The progestin (progesterone [P_4] + 20α-dihydroprogesterone [20α-OHP]) concentration in the culture medium was determined using RIA. All culture medium sample was diluted to 1 ml with distilled water and extracted with 4 ml diethyl ether. The extract was then radioimmunoassayed without chromatography by using a specific antibody to P_4 or 20α-OHP generated in our laboratory, as previously reported (21, 22). The range of linearity was between 50 and 1600 pg. The percent cross-reaction of the steroid with this antibody was determined in a manner similar to that described by Naito *et al.* (23).

Western blotting. The expression levels of rat FasL in luteal cells were determined by Western blotting using a specific antibody to rat FasL as previously reported by Tanaka *et al.* (24). Luteal cells were plated in 6-well plates at a density of approximately 9.38×10^5 cells/well in 7.5 ml culture medium and cultured for 24 h. After the culturing period, the culture supernatants were recovered and centrifuged to remove floated dead cells. The cell lysates resolved in 1 ml RIPA buffer (20 mM Tris, 1% SDS, 1% Triton-X100, 1 mM PMSF, 50 ng/ml leupeptin, pH 7.4) were centrifuged, and the supernatants were used for Western blotting. 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 RT with 2000-fold diluted anti-rat FasL antibody (C-20; Santa Cruz Biotechnology, Inc.) in PBS supplemented with 0.5% BSA and 25% Block Ace. After washing three times with PBS containing 0.1% Tween 20 for 30 min, the membrane was incubated for 30 min at RT with 1000-fold diluted peroxidase-conjugated goat anti-rabbit IgG (A0207; Vector Laboratories Inc.). The membranes were washed three times for 30 min with PBS containing 0.1% Tween 20, and the proteins recognized by the antibody were visualized by the ECL system (Amersham).

Immunostaining and TUNEL assay. The ovaries from cycling rats were immersed in 4% PFA in PBS, and sectioned at 6 μm, and placed on a silane-coated glass slide. The sections were immersed in 0.3% H_2O_2 in methanol and were preincubated with 4% skim milk for 90 min at RT. The slides were washed for 10 min 3 times, then the sections were incubated with rabbit anti-rat FasL antibody (C-178; Santa Cruz Biotechnology, Ltd.) (1:50) or anti-rat CD3 monoclonal antibody (1F4; Cerotec) (1:50) diluted with 1% BSA in PBS at 4°C overnight. Thereafter, these sections were incubated with biotinylated goat anti-rabbit IgG (1:500) or HRP-conjugated anti-mouse IgG (American Qualex Antibodies) (1:100) for 60 min at RT. In case of using the biotinylated antibody, the biotinylated antigen-antibody complex was achieved by using an Elite ABC-Peroxidase kit (VECTASTAIN). After washing, the sections were visualized by diaminobenzidine (DAB) in H_2O_2 solution and counter stained with 0.5% methylgreen in acetic acid buffer (pH 4.0).

To detect cells exhibiting DNA fragmentation in histological sections, we used a TACS *in situ* apoptosis detection kit (Trevigen, Inc.). Briefly, the slides were dipped in PBS for 10 min to rehydrate the sections, and we placed 50 μl of Proteinase K solution (diluted 1 μl of 1.0 ml stock into 50 μl of deionized water per sample treated) onto the sample for 15 min at RT to strip nuclei of the tissue. The sections were immersed in 2% H_2O_2 in methanol for 15 min at RT rinsed once with distilled deionized water, and covered with terminal deoxynucleotidyl transferase (TdT) buffer alone. Then, the TdT buffer containing biotinylated TdT dNTP, divalent cation (Co^{+2}), and TdT enzyme was added to the sections, and the slides were incubated in a humidity chamber for 60 min at 37°C. The reaction was terminated by transferring the slides into the stop buffer for 5 min at RT. Diluted Strep-HRP solution (streptavidin-HRP diluted to 1/500 in blue strep HRP diluent) was applied onto each sample for 10 minutes at RT. Blue Label solution was added to each sample, which was then



incubated for up to 7 min at RT. The slides were washed for 10 sec with deionized/distilled water 4 times. Thereafter, the slides were air dried and dehydrated twice for a few seconds in o-xylene, and finally the section was mounted.

Statistical analysis. The Student's t-test and Duncan's multiple range test were used for statistical evaluation of the results. Differences of $P < 0.05$ were considered statistically significant.

RESULTS

PRL Induces Apoptotic Cell Death in Luteal Cell Cultures

To confirm whether the *in vivo* function of PRL for induction of luteolysis at proestrus could be reproduced in a primary culture condition, the luteal cells were prepared from CB-154-treated rats on the day of proestrus for the culture study. Consistent with the data previously reported (10), the addition of PRL into a culture well for 24 h caused a significant decrease in the viability of luteal cells (Fig. 1B and C). Extensive internucleosomal DNA fragmentation was simultaneously observed in DNA specimens isolated from cultured luteal cells (data not shown; see (10)), and nucleic chromatin-condensation was detected *in situ*, which was characteristic of cells dying by apoptosis (Fig. 1A). Furthermore, the number of luteal cells attached to the plate was significantly reduced by PRL (65% of control; Table 1). These apoptotic effects of PRL were expressed from 3 h to 24 h after PRL addition (Fig. 1C) in a dose-dependent manner (Fig. 1B).

Apoptotic Effect of PRL Reduces Seroidogenic Activity of Luteal Cells

The cultured luteal cell preparations used in this study contained non-steroidogenic cells such as immune cells, endothelial cells, or fibroblasts (25). To determine which cell type(s) is/are responsible for the oligonucleosome formation and the reduction in viabil-

FIG. 1. PRL induces apoptotic cell death in luteal cell culture. A: Hoechst 33528 staining of cultured luteal cells. Luteal cells were cultured for 24 h in the presence of PRL or a PRL-free vehicle. At the end of incubation, cells were fixed and nuclei were stained with Hoechst 33528. (a): PRL (2 μg/ml), (b): control. The solid arrow indicates the apoptotic nuclei with chromatin condensation. B: Dose-dependent effect of PRL on the viability of cultured luteal cells prepared from CL at proestrous 1800 h. The donor rats were pretreated with CB154 to prevent the occurrence of a natural PRL surge. Luteal cells were cultured for 24 h in the presence of various concentrations of PRL. 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 ± SE, n = 4, * $P < 0.05$ vs control). C: Time-dependent effect of PRL on the viability of cultured luteal cells. Luteal cells were cultured for 24 h in the presence of 2 μg/ml PRL. The results are expressed as percentages of the average viability of the control cells cultured with the vehicle (mean ± SE, n = 4, * $P < 0.05$ vs control). Different letters indicate significant differences ($P < 0.05$).

TABLE 1

Effects of PRL on Progestin Output and Number of Steroidogenic Cells in Culture

	Progestin (P_4 + 20α-OHP)** (ng/ml supernatant)	No. of 3β-HSD-positive cells***/mm^2
Control*	165.1 ± 1.6	44.3 ± 2.3
PRL*	132.3 ± 6.8	19.5 ± 2.6
No. of experiments	n = 4	n = 6
P value	P < 0.01	P < 0.001
	No. of total cells***/mm^2	3β-HSD-positive/total cells (%)
Control	113.5 ± 5.2	39.2 ± 1.8
PRL	74.3 ± 6.2	26.3 ± 3.0
No. of experiments	n = 6	n = 6
P value	P < 0.001	P < 0.01

* Luteal cells were taken from proestrous donor rats pretreated with CB-154 and cultured for 24 h in chamber-slides with or without PRL (2 μm/ml).

** Supernatants were collected for RIAs of progesterone and 20α-dihydroprogesterone (20α-OHP).

*** Luteal cells were subjected to 3β-HSD activity staining, and the number of cells per mm^2 was counted. The values were expressed by the mean ± SE.

ity, the steroidogenic cells in culture were identified with a 3β-HSD activity staining. The number of 3β-HSD-positive cells per mm^2 in a culture well was significantly decreased by the addition of PRL, and interestingly the ratio of 3β-HSD-positive cells to the total number of cells was also decreased (Table 1). Chromatin condensation in apoptotic nuclei was observed almost exclusively among these steroidogenic cells (data not shown). Coinciding with this observation, we noted that the concentration of total amounts of progestin (P_4 + 20α-OHP) released into the culture medium was lower in culture wells to which PRL had been added (Table 1).

PRL Enhances the Expression of Fas Ligand in Cultured Luteal Cells

The expression of Fas has been confirmed in various tissues including the luteal tissue, and its physiological role in ovarian remodeling has been suggested (11–13, 18). Thus, Western blot analysis was made in either the supernatant of culture media or the cell lysate of cultured luteal cells for detecting FasL. The 26-kD bands corresponding to the soluble form of FasL (sFasL) were detected in both cell lysates and supernatants (data not shown). PRL distinctively increased the amount of sFasL protein in luteal cell lysates, though there was no observable change in sFasL protein levels in the supernatants (data not shown). The 40-kD bands corresponding to the membranous form of FasL (mFasL) were additionally detected on the same membrane. The amount of this mFasL in luteal cell lysates was increased by addition of PRL (Fig. 2A). We revealed that each samples nealy included equivalent protein, by using Coomassie brilliant blue staining (data not shown). To determine whether the luteal cells apoptosis induced by PRL stimulation is actually mediated by Fas/FasL system, we added anti-rat FasL antibody into the culture medium to prevent FasL function. PRL-induced luteal cells apoptosis was effectively suppressed by addition of the antibody (Fig. 2B). Although the antibody we used for this experiment was polyclonal antibody, they might contain some subtypes of antibody which could inhibit FasL fanction. These data strongly suggested that the candidate for intermediate signals at the downstream of PRL should be FasL protein.

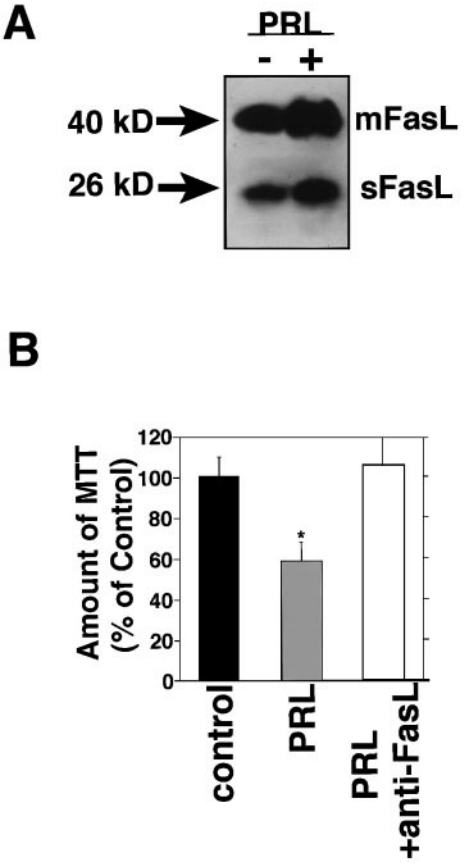


FIG. 2. A: PRL enhances the expression of FasL in luteal cells. Western blot analysis of FasL in the cultured rat luteal cells. Cell lysates from cultured luteal cells incubated with or without PRL (PRL + or −) were analyzed by immunobloting with anti-FasL antibody to detect soluble form (sFasL) and membranous form of FasL (mFasL). B: Addition of anti-FasL antibody attenuates PRL-induced luteal cells apoptosis. Luteal cells were cultured with or without anti-rat FasL antibody (5 μg/ml) for 24 hrs 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 in control cells. (mean ± SE, n = 4 * P < 0.05 vs control).

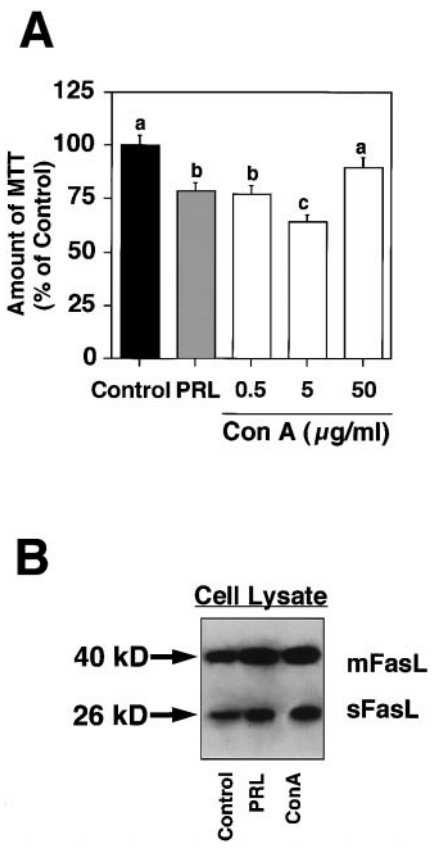


FIG. 3. ConA substitutes for the function of PRL in luteal cells culture. A: The apoptotic effect of PRL and Concanavalin A (ConA) on viability of cultured luteal cells collected from CL at proestrous 1800 h. The donor rats were pretreated with CB154 to prevent the occurrence of a natural PRL surge. Luteal cells were cultured for 24 h in the presence of PRL (2 μg/ml) or graded concentrations of ConA. 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 $\pm$ SE, n = 8). Different letters indicate significant differences ($P < 0.05$). B: Western blot analysis of FasL in the cultured rat luteal cells. Luteal cells were cultured for 24 h in the presence of PRL or ConA. Cell lysates were analyzed by immunoblotting with anti-FasL antibody to detect the soluble form (sFasL) and the membranous form of FasL (mFasL). Left lane: control (vehicle), middle lane: PRL (2 μg/ml), and right lane: ConA (5 μg/ml).

ConA Substitutes for the Function of PRL in Luteal Cell Cultures

Next we tried to determine which cell type in the luteal tissue expressed FasL as a result of PRL stimulation. Before addressing this problem directly, we hypothesized that the most probable cell type that expressed FasL and induced cell death in other types of luteal cells (e.g., steroidogenic cells) was the immune cell, such as the T-cell. If this is the case, concanavalin A (ConA), which is known as a T-cell specific activator and also an inducer of FasL (26), should have apoptotic effects on luteal cells similar to the effects of PRL. As shown in Fig. 3A, 0.5-5 μg/ml ConA induced apoptosis in luteal cells much as PRL did. The highest concentration of ConA (50 μg/ml), however, did not decrease the total number of viable luteal cells in culture. This apparent ineffectiveness may be caused by a mitogenic effect of ConA on T-cell proliferation. Since the luteal cell count in this study includes T-cells, the total number of cells could have remained the same as a result.

We noted that 5 μg/ml ConA enhanced the expression of both the soluble form and the membranous form of FasL in luteal cells in the same proportions as noted in a previous report where splenocytes were used (26) (Fig. 3B). These observations suggest that the source of FasL is ConA-sensitive immune cells in the luteal tissue.

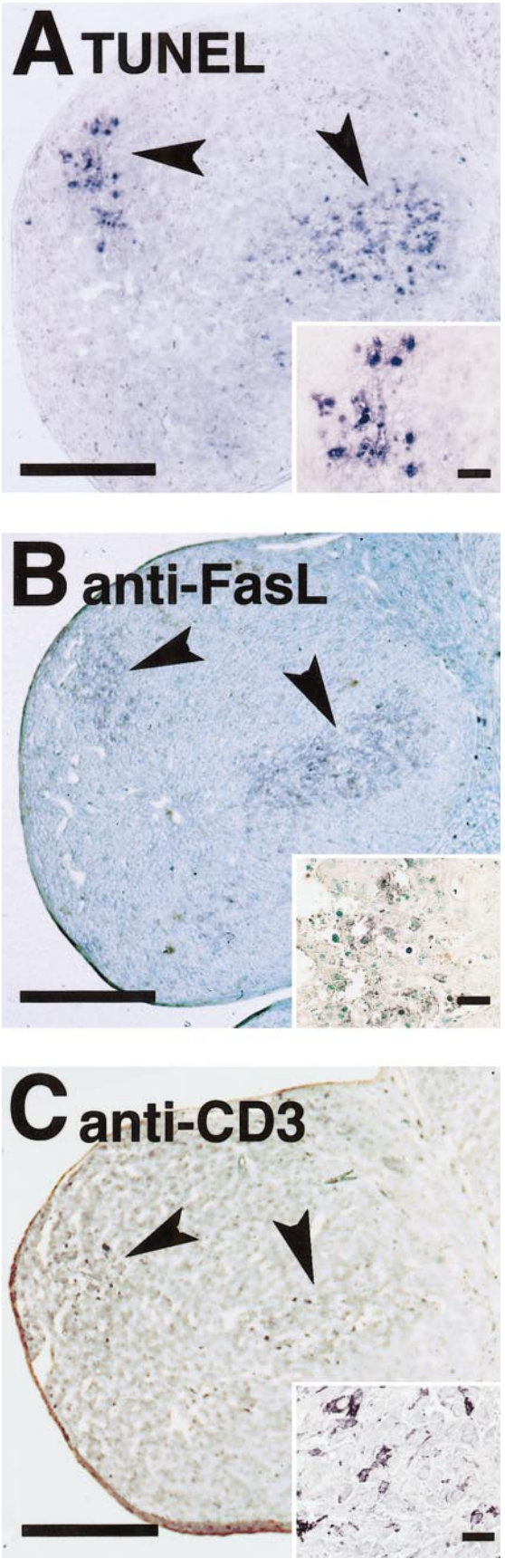
Localization of TUNEL-Positive and FasL-Expressing Cells in the Luteal Tissue

Based on the results described, we correlated the localization of T-cells immunohistochemically with that of either FasL-expressing cells or TUNEL-positive apoptotic cells using the luteal tissue obtained from intact cycling rats at the prostrous stage. We observed a few CLs in which TUNEL-positive cells assembled by forming clumps (Fig. 4A). The majority of positive signals of mFasL (Fig. 4B) and CD3 (Fig. 4C) were distributed within the clumps of TUNEL-positive cells. These data also indicated that the luteal immune cells are involved in luteolysis that depends on Fas/FasL function.

DISCUSSION

The present study suggests a novel fashion of cell death with the expression of an apoptotic molecule enhanced by PRL. An addition of PRL to cultured luteal cells was shown to reproduce an apoptotic cell death process *in vivo* (10), indicating that PRL could work on certain cell type(s) in a luteal cell culture preparation and directly initiate luteal cell apoptosis. The luteal cells in this study were harvested from proestrous rats that had been exempted from the endogenous PRL surge by a dopamine agonist. Because we observed a similar apoptotic effect of PRL on luteal cells prepared from diestrous rats (unpublished data), we concluded that a stage-specific endocrine environment *in vivo* is not involved in this apoptotic process initiated by PRL.

PRL was shown to increase the cell viability of luteal cells *in vitro*, if they were prepared from mid-pseudopregnant rats (10). Further, PRL has been recognized as a mitogen of T-cells, splenocytes and Nb2 lymphoma cells (27, 28) and thus, PRL can be expected to cause proliferation of lymphocytes in the luteal tissue. These observations imply that PRL is a survival factor rather than a death factor. Nevertheless,we observed in this study that apoptotic cell death was induced by PRL mainly in steroidogenic cells.The luteal tissue is composed of heterogenous cell populations (25). The function of steroidogenic cells may be modu-



lated by other non-steroidogenic cells such as endothelial and immune cells through their secretory activity of growth factors. In fact, we previously reported that TGF-β secreted by luteal macrophages was a prerequisite for full expression of steroidogenic activity of luteal cells (29, 30). FasL, which is a member of the TNF family, binds to Fas and induces apoptosis rapidly in Fas-expressed cells (16, 26). Because murine luteal cells are reported to express constitutively a large amount of Fas (12–14), those cells in the luteal tissue are considered to be ready to undergo apoptosis as soon as other cells in the luteal tissue, express FasL. Thus, we examined the relevance of FasL to PRL-induced apoptosis and found that PRL enhanced the expression of the membranous form of FasL (mFasL) in the cell lysate, but not the soluble form of FasL (sFasL) in the supernatant of luteal cell culture. Moreover, our result that the addition of anti-FasL antibody into medium inhibited PRL-induced apoptosis remarkably supported our idea that Fas/FasL system should mediate PRL-sensitive luteal regression. Based on these findings, the outline of a predicted mechanism for structural luteolysis could be described as follows: first, PRL binds its receptor on some cell type and induces the expression of mFasL on the surface of that cell type, and second, cells expressing mFasL interact with Fas-expressing steroidogenic cells and induce apoptotic cell death in steroidogenic cells.

A remaining question is what type of cells in the luteal tissue can express mFasL as a result of PRL stimulation. Before addressing this question directly, we first examined the effect of ConA on the occurrence of luteal cell apoptosis in order to confirm involvement of T-cells in this process. If this is the case, ConA could mimic the apoptotic process induced by PRL, because ConA induces proliferation and FasL-expression in T-cells (26, 31). In concert with our assumption, an addition of ConA to the luteal cell culture decreased the cell viability and enhanced FasL expression in the cell lysate of the luteal cell culture in a fashion similar to that of PRL. This is the first reported observation of ConA inducing apoptosis in cultured luteal cell preparation through the Fas/FasL system. IL-2 is reported to induce FasL expression in splenocytes (26). PRL's

FIG. 4. TUNEL-positive cells co-localize with FasL- and CD3-positive cells in CL. An intact rat at the proestrous stage was the donor. A, B, and C are adjacent sections. A: Histochemical localization of apoptotic cells detected by the TUNEL method (bar = 500 μm). Apoptotic cells assemble as clumps (arrow). The inset is a higher magnification of one of the clumps (bar = 20 μm). B: Immunohistochemical localization of FasL-positive cells in CL (bar = 500 μm). Most of the FasL-positive cells assemble within the clumps where the TUNEL-positive cells are assembled (arrow). The inset is a higher magnification (bar = 20 μm). C: Immunohistochemical localization of CD3-positive cells (bar = 500 μm). Most of the CD3-positive cells assemble within the clumps of TUNEL-positive cells (arrow). The inset is a higher magnification (bar = 20 μm).

mode of action for activing a FasL-expressing pathway in T-cells could be understood by comparing it to IL-2, because PRL and IL-2 receptors are members of the cytokine receptor family utilizing the Jak/STAT signaling pathway and are expressed on immune cells, including T-cells (32).

Finally, cytochemical analyses of the CL tissue in this study further supported the idea that T-cells are involved in the course of PRL-induced apoptosis. It was a rather unexpected finding to see that TUNEL-positive cells were not evenly distributed but rather assembled at particular portions, making "nests" in the CL. Immunohistochemical study indicated that FasL-positive cells were most frequently observed within these nests. In rodents, the membranous form of FasL, but not the soluble form (33), has a potent killer activity, so if the Fas/FasL system is involved in the mechanism of structural luteolysis, the direct cell surface interaction with target cells would be required. The co-localizations of TUNEL- and FasL-positive cells in CL tissue strongly suggest that the Fas/FasL cell death system is actually operating. The CD3-positive cells were detected in any regressing CLs in ovarian tissue specimens taken from proestrous rats (data not shown), and they also co-localized with TUNEL-positive cells and thus with FasL-positive cells. According to the similarity in size and shape of cells in adjacent sections, at least some CD3-positive T-cells appeared to co-express FasL.

In summary, we established an *in vitro* system mimicking the partial process of structural luteolysis *in vivo* induced by PRL. The structural luteolysis seemed to be initiated by apoptotic cell death in steroidogenic cells which was accompanied by a reduction in progestin secretion. This PRL-induced apoptosis depends at least in part on the Fas/FasL system. The Fas/FasL system is initiated by the induction of FasL expression brought on by PRL stimulation. This FasL is expressed in membranous form at least in part in CD-3-positive T-cells assembled in particular parts of CL tissue. This structural luteolysis process led by PRL is reproduced by ConA. Further characterization of these specific T-cells is underway.

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