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Caspase-3 Is a Pivotal Mediator of Apoptosis during Regression of the Ovarian Corpus Luteum

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Because caspase-3 is considered a primary executioner of apoptosis and has been implicated as a mediator of luteal regression, we hypothesized that corpora lutea (CL) derived from *caspase-3* null mice would exhibit a delayed onset of apoptosis during luteal regression, when compared with CL derived from wild-type (WT) mice. To test this hypothesis, ovulation was synchronized in immature (postpartum d 24–27) WT and caspase-3-deficient female littermates by exogenous gonadotropins. Individual CL were isolated by manual dissection, 30 h after ovulation, and placed in organ culture dishes in the absence of serum and growth factors. At the time of isolation (0 h) and after 24, 48, and 72 h in culture, the CL were removed and assessed for the presence of processed (active) caspase-3 enzyme and for apoptosis by multiple criteria. There was no evidence of active caspase-3 enzyme or apoptosis in either WT or caspase-3-deficient CL before culture. However, CL derived from the WT mice exhibited a time-dependent increase in the level of active caspase-3 and apoptosis during culture. By comparison, CL derived from caspase-3-deficient mice, cultured in parallel, failed to exhibit any detectable active caspase-3 and showed attenuated rates of apoptosis. To extend these findings derived from *ex vivo* culture experiments, ovaries were collected from WT and *caspase-3* null female littermates at 2, 4, or 6 d post ovulation, and the occurrence of apoptosis within the CL was analyzed. Whereas ovaries of WT mice had only residual luteal tissue at d 6 post ovulation, ovaries collected from caspase-3-deficient mice retained many CL, at d 6 post ovulation, that were similar in size to those observed in the early luteal phase of WT mice. Importantly, there was no dramatic increase in apoptosis in CL of caspase-3-deficient mice at any time point examined post ovulation, indicating that the involution process had indeed been delayed. In contrast, the levels of progesterone declined regardless of genotype. These data provide the first direct evidence that caspase-3 is functionally required for apoptosis to proceed normally during luteal regression. However, caspase-3 is not a direct mediator of the decrease in steroidogenesis associated with luteolysis. (*Endocrinology* 143: 1495–1501, 2002)

THE CORPUS LUTEUM (CL) is primarily responsible for the synthesis and release of progesterone, a hormone essential for the establishment and maintenance of pregnancy in mammals. In the absence of pregnancy, the CL will cease to synthesize progesterone, and the bulk of the luteal tissue will decrease in mass. This process, termed luteolysis, allows for the resumption of the estrous/menstrual cycle. The mechanism(s) by which steroid synthesis is inhibited and by which the structural involution of the tissue is initiated has not been fully elucidated. There is, however, evidence that luteal regression is, at least in part, mediated by the programmed cell death pathway of apoptosis in essentially all mammalian species examined to date (1–6).

Apoptosis is a physiological form of cell death that can be initiated by a number of stimuli, including activation of the so-called death receptors (*e.g.* Fas, TNF receptor-1) and serum or growth factor withdrawal (7–9). Once a cell receives a lethal stimulus, the program of apoptosis is then regulated and executed by members of the Bcl-2 and caspase families, respectively. The gene encoding Bcl-2 was discovered at a chromosomal breakpoint that predisposed individuals to development of B-cell lymphoma (10, 11). Since its discovery, numerous Bcl-2-related proteins have been isolated and characterized, with respect to their function in apoptosis. For example, some members inhibit apoptosis (Bcl-2, Bcl-x_L, Mcl-1, and Bcl-w), whereas others facilitate or induce apoptosis (Bax, Bak, Bad, Bid, Hrk/DP5, Mtd/Bok, and Bim) (12–14). Through their interactions at the level of mitochondria, Bcl-2 family members are considered to be the principal determinants of whether or not the execution of apoptosis proceeds via the activation of caspases (13, 15).

Of the 14 identified caspase family members in mammals, caspase-3 is unquestionably the most well-characterized enzyme. Activation of caspase-3, considered by many as a final executioner of the apoptotic cell death program, leads to rapid cleavage of a diverse spectrum of key structural and functional proteins in the cell. These targets for proteolytic attack include cytoskeletal components, signal transduction molecules, DNA repair enzymes, RNA splicing factors, nuclease activity-modulating factors, and nuclear matrix proteins (16, 17). Recent studies have shown that caspase-3 is expressed in the human CL (18), and have provided indirect evidence that caspase-3 is involved in luteal regression in several species (4, 19–22). However, all of these reports provide correlative data at best, thus raising the question of how functionally important caspase-3 is to the process of lute-

Abbreviations: CL, Corpus luteum (corpora lutea); KO, knockout; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end-labeling; WT, wild-type.

olysis. Therefore, the present study was designed to determine whether CL of caspase-3-deficient mice exhibit overt defects in luteolysis and whether these defects are, in turn, attributable to the inability of luteal cells to execute apoptosis.

Materials and Methods

Animals

Wild-type (WT) and caspase-3-deficient female mice (congenic C57BL/6) were generated by mating heterozygous male and female mice (23). Offspring were genotyped by PCR analysis of tail-snip genomic DNA that amplifies the region of the *caspase-3* gene targeted by homologous recombination (23). Ovulation was induced in all experiments, at d 24–27 post partum, by ip injection of 10 IU equine CG (Professional Compounding Centers of America, Houston, TX) followed by 10 IU human CG (Serono Laboratories, Inc., Norwell, MA) 46 h later. All animal protocols were reviewed and approved by the Massachusetts General Hospital institutional animal care and use committee and were performed in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Ex vivo culture

Corpora lutea were mechanically isolated, 30 h after induced ovulation, and cleaned of surrounding follicles and stromal tissue. Some CL were immediately frozen (for gel electrophoretic analysis of DNA integrity) or were fixed (for histology and histochemistry) in 10% neutral-buffered formalin, paraffin-embedded, serially sectioned (6 μm), and mounted on glass microscope slides. This group served as time 0 (no culture) data points. The remaining CL were cultured in serum-free conditions as previously described for neonatal mouse ovaries (24). Briefly, after isolation the CL were transferred onto nucleopore polycarbonate membranes (Transwell Membrane, 3.0-μm pore size, 24-mm diameter; Costar, Cambridge, MA) in Waymouth's MB752/1 culture medium (Life Technologies, Inc., Grand Island, NY) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1.3 μg/ml Amphotericin, and 0.23 mM pyruvic acid, and cultured for 24, 48, or 72 h, before freezing or fixation as described above. For histochemical assays, the largest diameter sections of the serially sectioned CL were used.

In vivo studies

To validate the induced luteal phase mouse model, ovaries and blood samples were collected from WT mice at 0.5, 1, 2, 3, 4, 5, and 6 d after induced ovulation (n = 3/time point). The ovaries were fixed in 10% neutral-buffered formalin, paraffin-embedded, serially sectioned (6 μm), and mounted, in order, on glass microscope slides. For *in situ* analysis of DNA fragmentation [terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end-labeling (TUNEL)], the largest diameter sections from each ovary were used. After determining the progesterone levels and incidence of apoptosis in the CL of WT mice, ovaries and blood samples were collected from caspase-3-deficient female mice (n = 3 mice/genotype) on d 2, 4, and 6 after induced ovulation (see above), for comparison.

Immunohistochemistry

Paraffin-embedded sections (6 μm) were analyzed by immunohistochemistry for the presence of cleaved (active) caspase-3, as previously described (21, 25). Briefly, after peroxidase quenching, sections were incubated with a 1:3,000 dilution of a rabbit polyclonal antibody that recognizes cleaved caspase-3 (CM1; IDUN Pharmaceuticals Inc., La Jolla, CA) (25, 26) for 1 h at room temperature. Sections were washed and then incubated with a 1:200 dilution of biotinylated goat antirabbit antibody (Calbiochem-Novabiochem, La Jolla, CA) for 1 h at room temperature. Sections were washed, incubated for 45 min at room temperature with horseradish peroxidase-conjugated streptavidin, washed, and then incubated with ice-cold 0.5-mg/ml 3,3′-diaminobenzidine with 0.03% hydrogen peroxide at room temperature, for antigen detection (brown reaction product). Sections were counterstained with hematoxylin and analyzed by light microscopy. Cells exhibiting the brown cytoplasmic staining from the colorimetric reaction were considered positive for active caspase-3. Negative controls, lacking the labeling enzyme, yielded no reaction product (data not shown). Similarly, ovaries collected from caspase-3-deficient mice had negligible (background) levels of staining. Sections of CL or ovaries from WT and caspase-3-deficient mice were processed in parallel within the same assay. The percent of caspase-3-positive cells was determined by counting CM-1-positive cells/total number of cells per field of view (600×) in sections from three separate CL from 3 different animals.

TUNEL

The occurrence of apoptosis in luteal and ovarian sections was assessed by monitoring the presence of DNA fragmentation *in situ*, as described previously (27). Slides were analyzed by conventional light microscopy after light counterstaining with hematoxylin. Cells exhibiting dark brown nuclear staining from the colorimetric reaction were considered positive for DNA fragmentation. Negative controls, lacking the labeling enzyme, yielded no reaction product (data not shown). Sections of CL or ovaries from WT and caspase-3-deficient mice were processed for TUNEL in parallel within the same assay. The percent of apoptotic cells was determined by counting TUNEL (brown reaction) positive cells/total number of cells per field of view (600×) in three separate CL from each ovary of three different animals.

DNA isolation, radiolabeling, and gel electrophoretic analysis

Genomic DNA was extracted from freshly isolated or cultured CL, quantitated and 3′-end-labeled with [α^{32}P]-dideoxy-ATP (3,000 Ci/mmol; Amersham Pharmacia Biotech, Inc., Piscataway, NJ) using terminal deoxynucleotidyl transferase (Roche Molecular Biochemicals, Indianapolis, IN) as described (27, 28). Radiolabeled samples were resolved through 2% agarose gels, and the extent of internucleosomal cleavage was qualitatively assessed by autoradiography.

Hormone analysis

Progesterone levels were measured in serum, according to the manufacturer's instructions, using a direct solid-phase enzyme-immunoassay (DRG Progesterone ELISA kit; ALPCO, Windham, NH). Before the assay, the serum samples were extracted with 6.6 vol ethyl ether; the extracts were dried under nitrogen and reconstituted in zero standard control serum provided by the manufacturer. Sera from WT and caspase-3-deficient mice were assayed in duplicate in the same assay. Progesterone concentration was determined by absorbance, at 450 nm, against a standard curve.

Data presentation and statistical analysis

Each experiment was independently replicated 3 times with different mice in each experiment. Data shown are representative of results obtained in the replicate experiments. For quantitation of immunohistochemical data, the total number of positive cells was determined in at least 3 fields of view per CL or ovary and calculated as a percent of the total number of cells per field of view. An average of 500 cells was counted per field of view. The results are expressed as the percent mean ± SEM. Quantitative data generated by RIA of progesterone levels are expressed as the mean ± SE. Statistical significance was determined by either *t* test or one-way ANOVA followed by Duncan's new multiple-range test. A *P* value less than 0.05 was considered statistically significant.

Results

Validation of an ex vivo model to study apoptosis in intact CL

To facilitate studies of the mechanisms underlying the activation of luteal cell death, without sacrificing the three-dimensional architecture or cell-to-cell contact of an intact CL, initial experiments were conducted to characterize the

occurrence of apoptosis in single CL maintained *ex vivo* under hormone- and growth factor-free conditions. Thirty hours after gonadotropin-induced ovulation, there was no evidence of active caspase-3 enzyme in CL freshly isolated from either WT (Fig. 1A) or caspase-3-deficient mice (Fig. 1B). However, processing of procaspase-3 to the active form became evident in CL derived from WT mice after 48 h in culture (Fig. 1E) and was widespread after 72 h in culture (Fig. 1G). By comparison, and as anticipated from the genotype, CL isolated from caspase-3-deficient mice had only light cytoplasmic staining of single cells observed on occasion at 48 and 72 h (Fig. 1, F and H). A significant increase ($P < 0.0004$) in the percent of CM-1-positive cells was observed in the CL of WT mice at 48 h (31.0 ± 1.15%), compared with the percent of CM-1-positive cells in CL of caspase-3-deficient mice at the same time point (4.6 ± 0.08%).

The onset of DNA cleavage is delayed in caspase-3-deficient CL ex vivo

Regardless of genotype, CL analyzed immediately after isolation were indistinguishable, by all immunohistochemical and biochemical assessments of apoptosis (Figs. 1 and 2). The mean number of TUNEL-positive cells increased, over time, in the cultures of CL derived from WT mice (Fig. 2; A, C, E, and G). At 48 h, there was a significant increase ($P < 0.005$) in the percent of TUNEL-positive cells observed in the CL derived from WT mice (32.6 ± 3.4% TUNEL-positive

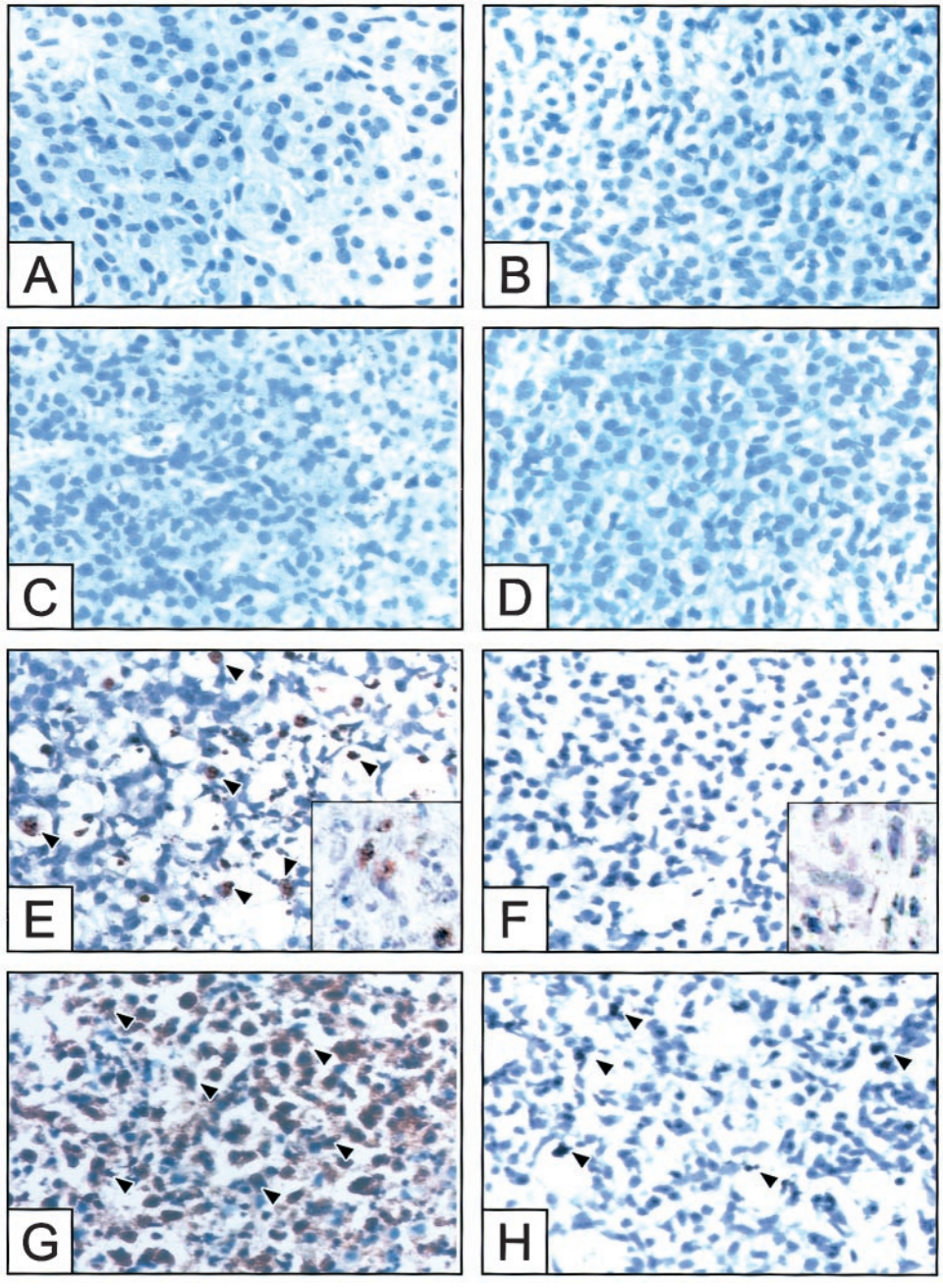

FIG. 1. Active caspase-3 in cultured CL. Active caspase-3 was identified by immunohistochemistry using CM1 antibody in sections of CL that had been cultured in serum-free conditions for up to 72 h. Shown are: CL from a WT mouse (A) and CL from a caspase-3-deficient mouse (B) immediately after isolation (0 h); CL from a WT mouse (C) and CL from a caspase-3-deficient mouse (D) after 24 h culture; CL from a WT mouse (E) and CL from a caspase-3-deficient mouse (F) after 48 h culture; and CL from a WT mouse (G) and CL from a caspase-3-deficient mouse (H) after 72 h culture. Original magnification, ×600. The *insets* in E and F represent a high magnification (original magnification, ×1000) demonstrating the presence or absence of CM-1-positive cells. Active caspase-3 was observed in WT mice after 48 and 72 h in culture (*dark brown* reaction). Medial sections were obtained from CL from three independent mice for analysis. Photomicrographs shown are representative of identical results in at least three separate experiments. *Arrows* indicate examples of CM-1-positive staining.

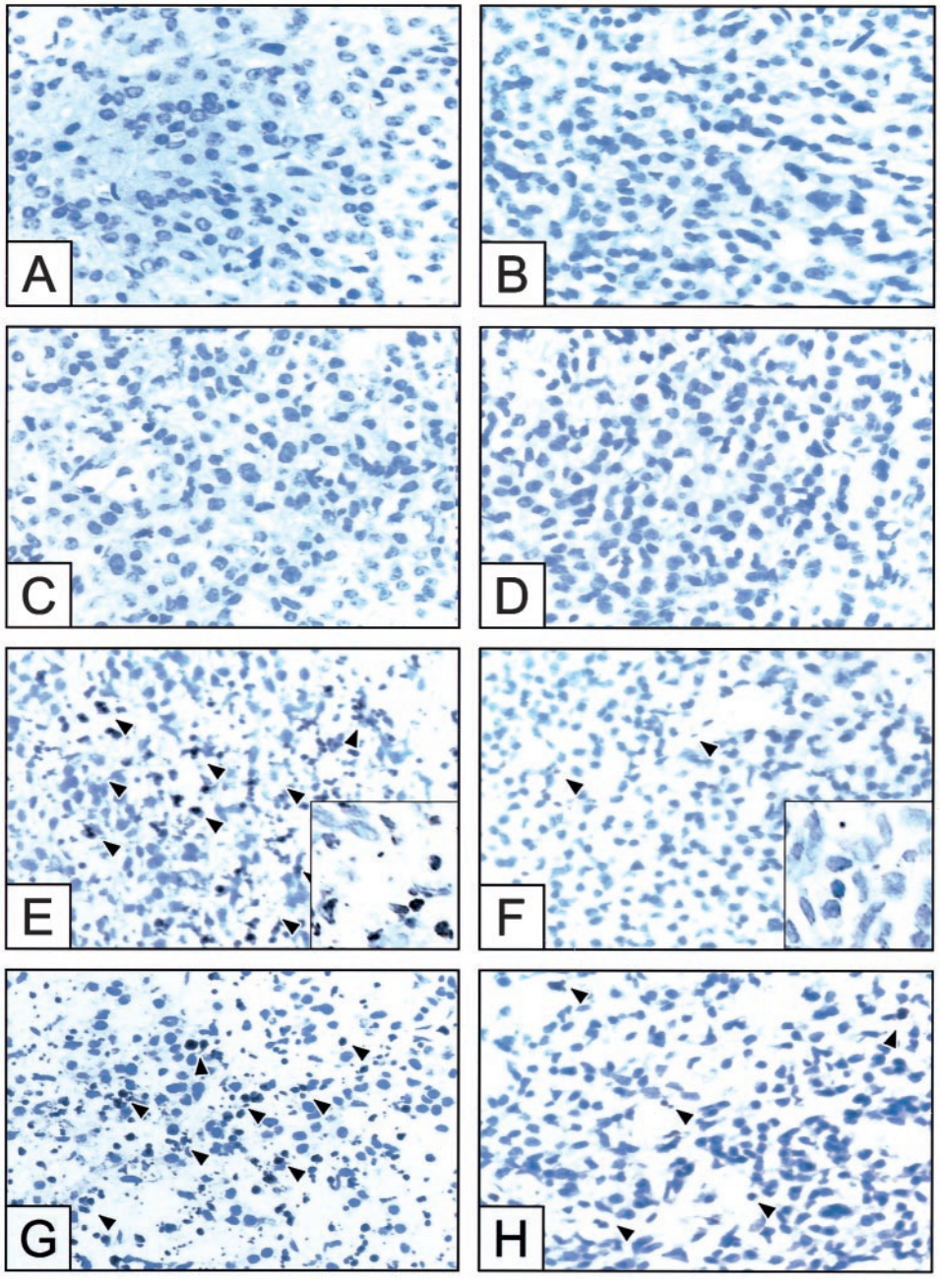

FIG. 2. Histochemical assessment (TUNEL) of apoptosis *in vitro*. Time-related appearance of *in situ* DNA fragmentation in sections of CL immediately after isolation, 0 h (A, WT; B, *caspase-3* KO), and after 24 h (C, WT; D, *caspase-3* KO), 48 h (E, WT; F, *caspase-3* KO), and 72 h of culture (G, WT; H, *caspase-3* KO mice). TUNEL-positive cells (see *dark brown* reaction products, E and G) were evident in WT mice. Original magnification, ×600. *Arrows* indicate examples of TUNEL-positive staining. The *insets* in E and F represent a high magnification (original magnification, ×1000) emphasizing the presence or absence of TUNEL-positive cells.

cells; Fig. 2E), compared with the CL derived from caspase-3-deficient mice (1.04 ± 0.3% TUNEL-positive cells; Fig. 2F). After 72 h in culture without hormonal support, the cells within the CL derived from the WT mice displayed all the expected features of apoptosis, including cellular condensation and detachment, DNA cleavage, and formation of large numbers of apoptotic bodies (Fig. 2G). Precise counts of TUNEL-positive cells were impossible because of the high levels of cellular debris in the CL of the WT mice. By comparison, all of these events were either markedly attenuated or absent in CL isolated from caspase-3-deficient mice cultured in parallel (Fig. 2, B and D, F, H). Because TUNEL alone is not always conclusive of apoptosis, electrophoretic analysis of low-molecular-weight DNA was performed. The attenuated onset of apoptotic cell death in CL collected from *caspase-3* null mice was further reflected by gel electrophoretic analysis of DNA integrity (Fig. 3).

In vivo regression of CL is delayed in caspase-3-deficient mice

To verify that the delayed onset of apoptosis, observed in the CL derived from caspase-3-deficient mice and cultured *ex vivo*, accurately reflected that which occurs during luteal regression *in vivo*, we analyzed CL in ovaries of WT and caspase-3-deficient mice at 2, 4, and 6 d after induced ovulation. Two days after ovulation induction, there was no evidence of apoptosis in any CL, regardless of genotype (data

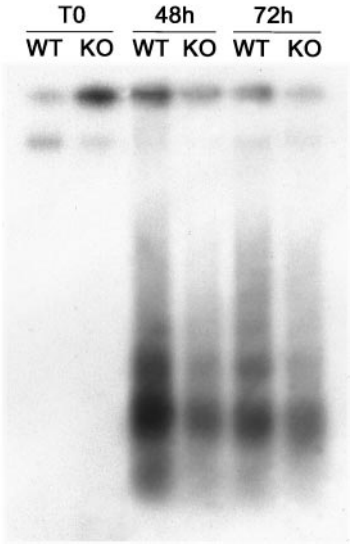

FIG. 3. Internucleosomal DNA fragmentation. DNA was isolated from CL derived from WT or caspase-3-deficient mice after time in culture, end labeled, electrophoresed through agarose gels (250 ng/lane), and the gel subjected to autoradiography for qualitative assessment. T0, Time zero.

not shown). By 4 d post ovulation, ovaries of WT mice contained several CL with large numbers of TUNEL-positive cells (Fig. 4, A and C). In contrast, only a few, if any, TUNEL-positive cells were detected in *caspase-3* null CL collected in parallel at d 4 post ovulation (Fig. 4, B and D). Based on counts of TUNEL-positive cells in the CL of WT and caspase-3-deficient mice collected on d 4, the number of TUNEL-positive cells/total number of cells per field of view was significantly higher ($P < 0.003$) in CL of WT mice (14.75 ± 2.14%), when compared with CL of caspase-3-deficient mice (1.3 ± 0.3%). In keeping with these data that suggest that loss of caspase-3 function delays involution of the CL, 6 d after ovulation, ovaries collected from WT mice contained only remnants of the CL (Fig. 4E). However, caspase-3-deficient ovaries still retained several large CL with a only few TUNEL-positive cells (Fig. 4F).

Serum levels of progesterone do not differ in WT and caspase-3-deficient mice

Levels of progesterone in serum of WT mice increased in response to human CG-induced ovulation and peaked on d 1 (6.38 ± 1.1 ng/ml) post ovulation. The levels declined precipitously thereafter, dropping to concentrations of less than 1 ng/ml by 4 d post ovulation (Fig. 5). There was no significant difference ($P > 0.05$) in the levels of progesterone in the caspase-3-deficient [knockout (KO)] mice, when compared with the progesterone levels in WT mice, over time, at either d 4 (WT, 1.99 ± 1.19 ng/ml; KO, 0.85 ± 0.03 ng/ml) or d 6 (WT, 0.9 ± 0.09 ng/ml; KO, 0.82 ± 0.00 ng/ml).

Discussion

Although apoptosis can be induced by a diverse spectrum of stimuli, many of the downstream mediators of apoptosis are evolutionarily conserved among species. For example, though TNFα and Fas ligand bind distinct membrane receptors, both have the ability to promote caspase-3-mediated apoptosis (29). Moreover, caspase-3 can be activated by more than one signaling pathway coupled to these so-called death receptors. Whether or not apoptosis is induced by an activated death receptor or an alternative apoptotic stimulus, caspase-3 is considered a common mediator of apoptosis (30, 31).

Whereas evidence of caspase-3 expression and activity has been shown in regressing CL of many species (18–20), it remains to be established whether caspase-3 is needed for apoptosis and structural involution associated with luteal regression. Previous studies designed to evaluate the possible role of caspase-3 in the CL have relied heavily on relatively pure or mixed luteal cell populations dispersed in monolayer cultures (21, 22). These studies have demonstrated that caspase-3 is present and activated coincident with apoptosis. Our first series of experiments, using intact CL to maintain the normal three-dimensional structure and cell-to-cell interactions of the CL, revealed a time-dependent onset of apoptosis in CL derived from WT mice, as assessed by both morphological and biochemical parameters. Furthermore, the increase in apoptosis was coordinately associated with an elevation in the level of processed (active) caspase-3 enzyme. These correlative results, which further strengthen the hypothesis that caspase-3 is involved in luteal cell death, were reinforced by our findings derived from comparing the onset of apoptosis in cultured CL collected from WT and *caspase-3* mutant female mice. Though freshly isolated CL of WT and *caspase-3* null mice were histologically and biochemically similar, the occurrence of apoptosis after *in vitro* culture was markedly delayed by caspase-3 deficiency.

To confirm and extend these findings, we next tested the importance of caspase-3 to luteal regression *in vivo*. As a model, prepubertal female mice were treated with exogenous gonadotropins to induce ovulation to ensure that no residual luteal tissue from a previous estrous cycle would be present. Under these experimental conditions, the CL of WT mice maintained their structural integrity for approximately 4 d, at which time there was an easily discernible decrease in overall luteal mass concomitant with the appearance of many TUNEL-positive (apoptotic) cells. By d 6 post ovulation, only remnants of the CL were visible. From a functional standpoint, the levels of progesterone increased after ovulation, reaching peak levels at approximately 30 h after luteal formation. Serum progesterone levels then declined dramatically, well before the onset of structural involution, as determined by TUNEL analysis of apoptosis.

In contrast to these *in vivo* observations of luteal growth and regression in WT mice, there was little to no evidence of TUNEL-positive cells in the CL of the caspase-3-deficient mice at 4 d post ovulation. Furthermore, luteal mass was clearly maintained by caspase-3 deficiency, as long as 6 d after ovulation, with only a few TUNEL-positive cells becoming detectable. This defect in the temporal onset of apoptosis in caspase-3-deficient mice *in vivo* was similar to that observed using the *ex vivo* culture model. Interestingly, the levels of progesterone in *caspase-3* null mice did not differ from their WT siblings at any time point examined post ovulation, despite the maintenance of CL mass caused by

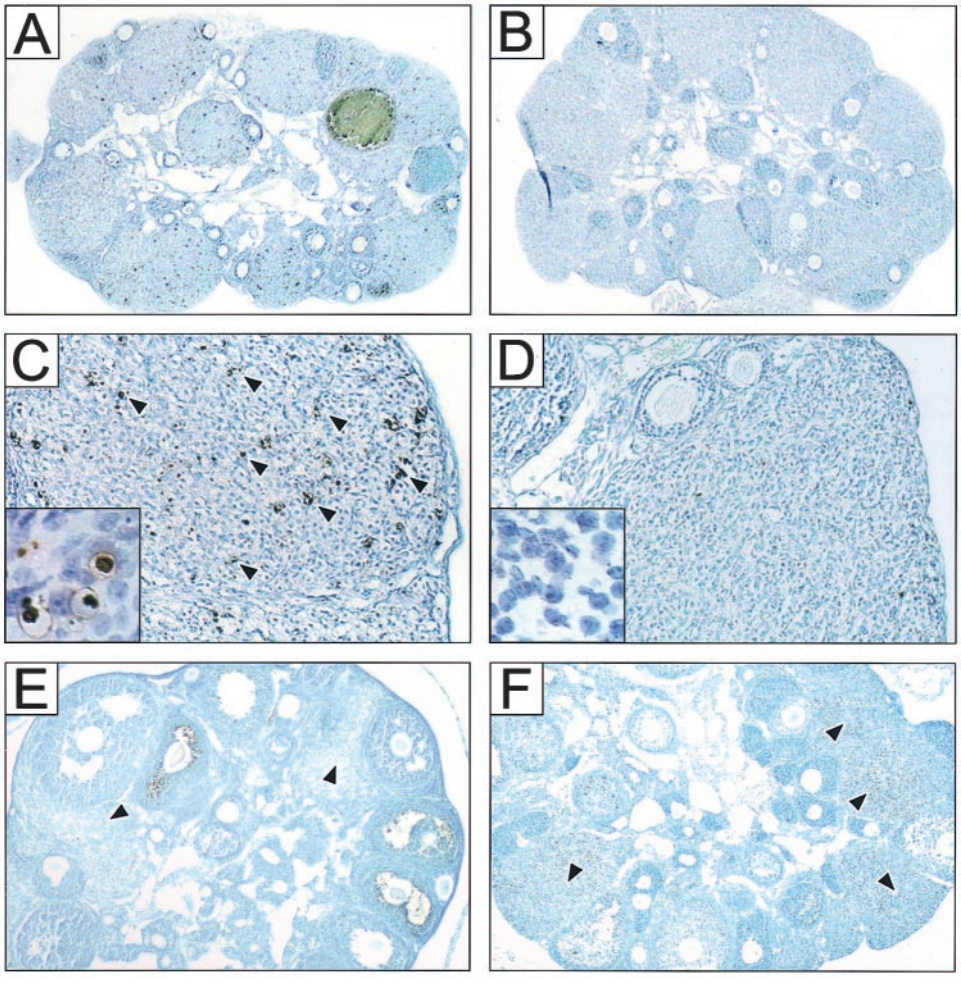

FIG. 4. Histochemical assessment (TUNEL) of apoptosis *in vivo*. Apoptotic cells were detected in ovaries derived from WT (A and C) and caspase-3-deficient (B and D) mice, 4 d post ovulation. Original magnification for A and B, ×100. C (ovary from WT mouse) and D (ovary from caspase-3-deficient mouse) are a higher magnification of A and B, respectively (×400). *Dark brown* reaction products denote TUNEL-positive cells (see *arrows*). The *insets* in C and D represent a high magnification (original magnification, ×1000), demonstrating the presence or absence of TUNEL-positive cells. E (WT) and F (caspase-3-deficient mice) represent ovaries at 6 d post ovulation. *Arrows* in E highlight the regressed CL in WT mice, and *arrows* in F highlight the intact CL in caspase-3-deficient mice.

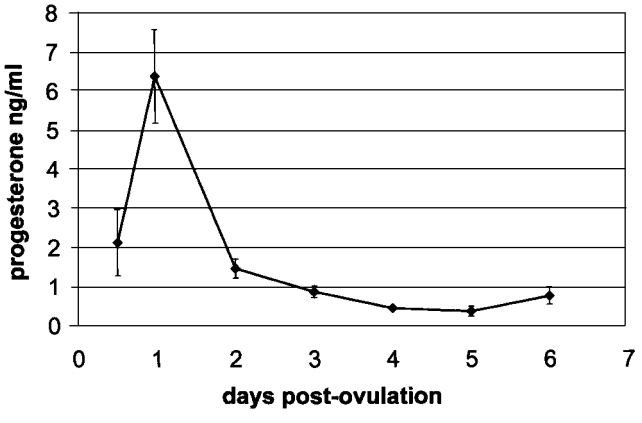

FIG. 5. Serum progesterone levels in mice after induced ovulation. Blood was collected from WT mice, over time, after gonadotropin-induced ovulation, and serum was isolated. The concentrations of progesterone were measured by ELISA as described in *Materials and Methods*. The data represent the mean ± SE (n = 3/time point).

caspase-3 loss-of-function. Such findings add important insight into mechanisms responsible for luteal regression, because the relationship between the induction of luteal cell death to the loss of steroidogenic capacity has remained unsettled. Indeed, based on our observations herein, it is likely that steroidogenic cells of the CL lose their capacity to produce progesterone via mechanisms independent of those responsible for executing apoptosis during structural involution.

It was previously reported that a reduction in progesterone may accelerate the apoptotic process in bovine luteal cells (32, 33). However, in the present murine model, the onset of apoptosis was delayed by the lack of caspase-3 despite the fact that progesterone had decreased. Because the functional and structural components of luteal regression can be separated temporally and mechanistically, at least in the mouse, the potential may exist to rescue a functionally deficient CL before its structural demise. This could be helpful clinically in cases of luteal insufficiency, which can lead to spontaneous abortions and/or irregular menstrual cycles (34). Interestingly, we have observed a number of reproductive anomalies associated with the caspase-3-deficient female mice, including failure to conceive and/or establish pregnancy. If the female becomes pregnant, the litter sizes are small in number, and it is not uncommon to have birth of nonviable offspring (unpublished data). Whether or not these abnormalities are attributable, entirely or in part, to the disrupted luteal regression that results from caspase-3 deficiency remains to be established. Future experiments will also be needed to characterize whether or not other presumed mediators of apoptosis may influence the loss of

steroidogenic function associated with luteolysis. Collectively, these studies may help to further define relationships between steroidogenisis and apoptosis susceptibility in the CL, and whether luteal cells rescued from apoptosis can regain steroidogenic capacity.

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