Programmed Cell Death in Preovulatory Ovine Follicles

William J. Murdoch

Department of Animal Science, University of Wyoming, Laramie, Wyoming 82071

ABSTRACT

Apoptosis is a mode of physiological cell death that occurs during tissue regression and remodeling; it is characterized by macromolecular protein synthesis, oligonucleosomal fragmentation, nuclear condensation, cytoplasmic shrinkage, and dissociation of cell-to-cell junctional complexes. Direct in situ fluorescence detection of digoxigenin end-labeled genomic DNA was used as a marker of nuclear apoptosis within preovulatory ovine follicles and contiguous ovarian tissues. As ovulation approached, there was a progressive increase in apoptotic cells within the ovarian surface epithelium, tunica albuginea, and apical follicular wall. At the avascular site of rupture, follicles were devoid of ovarian surface and granulosa epithelia. Granulosa cell dispersion within the basal region of preovulatory follicles was not associated with apoptosis. Discrete physico-chemical interactions between preovulatory follicles and the ovarian surface are evidently a prelude to programmed cell deletion and ovulation.

INTRODUCTION

The preovulatory surge in secretion of pituitary gonadotropins initiates a complex series of interactive ovarian events that compromise tissue integrity and lead to ovulation. The ruptured follicle is consequently transformed into the CL.

Dramatic alterations in organ morphology are often associated with a programmed process of active physiological cell death or apoptosis. Early-stage apoptosis is distinguished by endonuclease activation, chromatin degradation, and nuclear condensation. Apoptotic cells shrink and lose contact with their neighbors. Residual bodies are typically resorbed by adjacent epithelial cells or resident macrophages. Cells undergoing apoptosis may completely disappear within a few hours [1-6]. Apoptosis has been implicated in the mechanisms of follicular atresia [7-11] and luteal involution [12-14].

The objective of the present study was to determine whether or not apoptosis is a potential determinant of ovulation. It was hypothesized that programmed cell death contributes to dissolution of the follicular wall and rupture. In situ labeling of fragmented DNA was used as a diagnostic measure of ovarian apoptotic cell death in sheep. Immunostaining of apoptotic cells is a very sensitive technique to monitor biological reactions that might be undetectable by standard DNA ladder assays of entire tissues.

MATERIALS AND METHODS

Mature western range ewes were penned daily with vasectomized rams and observed for behavioral estrus. The first day of estrus was considered Day 0. On Day 14 of the estrous cycle, animals received i.m. an injection of 10 mg prostaglandin F_2alpha (Upjohn Co., Kalamazoo, MD); this was followed 36 h later with 5 μg Des-(d-Ala^6)-Gly^10 GnRH ethylamide (GnRH; Sigma Chemical Co., St. Louis, MO). These treatments synchronize the timing of luteal regression and the onset of the preovulatory surge of gonadotropins. The largest follicle in the pair of ovaries consistently ovulates approximately 24 h after administration of GnRH [15].

Midventral abdominal laparotomies were performed through use of aseptic technique under i.v. sodium thioptal anesthesia. Ovaries with the dominant follicle were surgically removed at 0, 8, 16, and 24 h after the GnRH injection. Six animals were included in each group. A block of ovarian tissue containing the follicle of interest and a portion of nonfollicular-associated ovary were excised with a single-edged razor blade and fixed by immediate immersion in 10% neutral buffered formalin. After 4 h, follicles were cut into apical and basal components, and tissues were placed in fresh fixative for an additional 44 h. Special care was taken not to handle the surface of the ovaries during removal or preparation for histology.

Fixed tissues were washed in PBS solution, dehydrated in a graded series of ethanol, cleared, and infiltrated with paraffin. Embedded tissues were serially sectioned at 5 μm, deparaffinized, rehydrated, and stained with hematoxylin and eosin (H & E) or processed for immunostaining of apoptotic cells. Sections stained with H & E were inspected under an Olympus Vanox (Olympus Optical Co., Tokyo, Japan) light microscope.

Apoptotic cells were detected by means of an Oncor (Gaithersburg, MD) S7110 ApopTag kit according to the instructions of the manufacturer. Briefly, exposed 3'-OH ends of DNA fragments were labeled with digoxigenin-11-d uridine triphosphate by terminal deoxynucleotidyl transferase (TdT) catalysis. Incorporated nucleotide heteropolymers were localized with antidigoxigenin Fab-fluorescein isothiocyanate. Sections stained with H & E were inspected under an Olympus Vanox (Olympus Optical Co., Tokyo, Japan) light microscope.

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Correspondence. FAX: (307) 766-5098.
FIG. 1. Representative photomicrographs of ovarian cross sections (H & E). A) Follicular wall (granulosa + theca) at 16 h (× 400). B) Apical granulosa layer at 16 h; exemplary pyknotic cells are designated with arrows—some cells were shed into the follicular antrum (a) (× 200). C) Basal granulosa at 16 h; mitotic figures (arrow) were often noted within the basal granulosa layer of preovulatory follicles (× 400). D) Desquamated avascular stigma of an unruptured follicle (os; ovarian surface) (× 200). E) Follicular rupture site (*) (× 200). F) Ovarian surface epithelium (ose) at 24 h just beyond the follicular boundary (arrow) (× 200).
of six randomly selected areas from sections of each tissue sample were photographed (40-sec exposure; Ektachrome 400 HC; Eastman Kodak, Rochester, NY) under an Olympus BH-2 microscope equipped with a reflected light fluorescence attachment. Numbers of reactive (intensely stained) cells within a particular zone (surface epithelium, tunica albuginea, apical theca, apical granulosa, basal theca, basal granulosa) were counted (×400). Data for surface epithelium and granulosa cells are expressed as a function of total cells represented (inclusive cell numbers could not be accurately determined from heterogenous interstitial tissues).

Subsample data for individual animals were averaged. Mean contrasts due to time of ovariectomy were made by one-way analysis of variance and protected least significant difference. Paired means within time were compared by Student's t-test. Differences were considered significant at p < 0.05.

RESULTS

A rupture point (n = 2) or a definitive avascular ovulatory stigma (n = 4) was present on follicles collected at 24 h after administration of GnRH. At 8 and 16 h there was evidence of preovulatory follicular swelling (antral fluid accumulation), but not of imminent rupture.

Dispersion of granulosa cells was evident among apical and basal granulosa cells by 16 h (Fig. 1, A-C). Pyknotic granulosa cells were abundant at the follicular apex (Fig. 1B). The supportive basement membrane of the granulosa layer was difficult to discern in follicles obtained at 16 (Fig. 1C) and 24 h. It appeared at 16 h that contracted apical granulosa cells had lost contact with their neighbors (Fig. 1B). Cytoplasmic shrinkage of basal cells was not perceptible; dissociation was presumed to be the result of dissolution of cell-cell junctions (Fig. 1C). Granulosa cells were scarce or virtually absent, and the thecal layer was disrupted along the apical wall immediately apposed to the site of impending ovulation or rupture (Fig. 1, D and E); no attempt was made to quantify apoptotic cells from these sections.

Epithelial cells were completely lacking over the ovarian surface juxtaposed to periovulatory follicles (Fig. 1D). Some loss of surface epithelium was observed at 16 h. Follicle-associated cells had apparently been shed in vivo. This phenomenon did not extend to the ovarian surface in general. Denuded areas were abutted by squamous cells (normal epithelium was cuboidal to low pseudostratified columnar) (Fig. 1F).

There were temporal increases in apoptotic cells localized within surface epithelia, tunica albuginea, apical theca,
and granulosa. Corresponding changes were not noted within the basal follicular wall (Figs. 2 and 3) or surrounding interstitium (data not shown).

**DISCUSSION**

To my knowledge, this is the first report indicating that apoptosis within preovulatory ovarian tissues could be a contributing factor in the biomechanics of in vivo ovulation. Evidence of nuclear DNA fragmentation was initiated as early as 8 h after induction of the LH surge along the ovarian surface adjacent to preovulatory ovine follicles. This implies that contact-mediated interactions occur between the ovarian surface and a follicle destined to ovulate. Preferential growth of preovulatory follicles presumably affords them the opportunity to come into close proximity with the ovarian surface. Indeed, in certain species ovulation is restricted to an area (fossa) covered by typical surface epithelium [16]. Degenerative changes at the putative site of follicular rupture in rabbits [17], mice, and hamsters [18] begin with the surface epithelium and move inward.

GnRH and its agonistic analogues have been reported to induce apoptosis in rat follicles [19]; in this species, direct ovarian effects of GnRH have been observed [20]. However, attempts to detect GnRH receptors or direct actions thereof in ruminant ovaries have been negative [21–23]. It would seem that apical follicular cell death in the sheep is the result of localized gonadotropin-mediated events.

Prostaglandins are candidate intrafollicular agents of apoptosis. Prostaglandin \( E_2 \) was found to be a potent apoptotic stimulus in primary cultures of sheep ovarian surface epithelium [24]. Initial anatomical evidence of DNA fragmentation (present study) coincides with intense prostaglandin endoperoxide synthase gene expression in epithelial cells overlying preovulatory ovine follicles [25] and with the peak in follicular wall prostaglandin \( E_2 \) accumulation [26, 27]. It is well known that inhibition of follicular prostaglandin biosynthesis blocks ovulation in mammals [28]. Treatment of sheep with indomethacin during the preovulatory period negated granulosa cell-cell dissociations [29] and surface epithelial degeneration [24, 30]. Apparently, dispersion of granulosa cells per se (a presumptive index of apoptosis) was unrelated to whether they perished (apex) or not (base).

A lack of blood flow into the stigma of follicles [31] might trigger localized apoptosis. Depletion of serum (e.g., growth) factors is evidently an initiating determinant in the death of oxygen-deprived cardiomyocytes [6]. According to this scenario, cells are primed to die upon removal of an apoptosis-inhibiting element. Steroid withdrawal provokes apoptotic cell death in estrogen-dependent mammary and androgen-dependent prostate tumors [32]. There was a precipitous decrease in follicular estradiol-17β and testosterone after the onset of the preovulatory gonadotropin surge in ewes [33].
An involvement of apoptosis in follicular dissolution does not necessarily preclude a role for lytic cellular damage. In contrast to apoptosis, necrosis is a nongenomic process caused by injury. It is exemplified by loss of plasma membrane integrity and inflammation [1]. Ischemia would presumably lead to secondary tissue necrosis, which might aid in follicular weakening. Infiltrative inflammatory cells also may participate in periovulatory processes [34, 35]. Moreover, nonspecific DNA degradation can occur in necrosis [20]. Morphological observations of cell shrinkage and pyknosis support the premise that apoptosis is a factor in the ovulatory process of sheep. The in situ method utilized in the present study detects apoptotic cells with a >10-fold higher sensitivity than it does necrotic cells [36].

Finally, physical forces may combine with selective cell elimination to assure tissue thinning and follicular rupture. Perhaps retraction of the basal theca brought about by follicular swelling and/or contractility causes the opposing wall to recede from the ovarian surface.

REFERENCES