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In Situ Localization of Apoptosis in the Rat Ovary during Follicular Atresia[1]

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ABSTRACT

Apoptosis is a type of physiologic cell death that occurs in many tissues and may be regulated by peptide growth factors. Recent studies indicate that apoptosis occurs in the ovary during follicular atresia in several animal species, including the rat, pig, chicken, baboon, and rabbit. The purpose of this study was to demonstrate, through in situ identification of apoptotic cells in intact ovarian sections, the sites in which apoptosis occurs in the rat ovary in different functional states. We evaluated the presence of apoptosis in three models: immature rats, eCG-treated rats and adult cycling rats. Paraffin ovarian sections were pretreated with proteinase K and then end-labeled with biotinylated deoxyuridine triphosphate (dUTP) by incubation with the enzyme terminal deoxynucleotidyl transferase (TDT). They were then stained through use of avidin-conjugated peroxidase with 3,3′-diaminobenzidine as the substrate. Healthy antral and preantral follicles had no staining. The nuclei of granulosa cells of preantral and antral atretic follicles were positively stained in all the animal groups. Scattered theca cells were also stained. Stromal cells were consistently negative. Positive controls were sections pretreated with DNase I; these displayed intense staining of all nuclei. Negative controls, in which either terminal TDT or its biotinylated substrate was omitted, were appropriately negative. This study represents a systematic analysis of apoptosis in the rat ovary at different functional stages and supports the hypothesis that apoptosis is involved in the process of follicular atresia.

INTRODUCTION

Apoptosis is a type of physiologic cell death that can be viewed as the antithesis of mitosis in the regulation of tissue homeostasis [1, 2]. It affects single scattered cells in the midst of living tissues, without eliciting an inflammatory reaction, and sometimes is dependent on the availability or withdrawal of growth factors and hormones. Initially described in connection with metamorphosis and embryogenesis [3], apoptosis is now recognized as a widespread physiologic phenomenon that participates in the remodeling of adult tissues [2].

Apoptotic cells are recognized by a characteristic fragmentation of their DNA into oligonucleosomes that are multiples of 180–200 bp. This fragmentation, which gives a typical ladder pattern on gel electrophoresis, occurs by the activation of specific endonucleases as opposed to the random DNA fragmentation of cell necrosis [4]. Apoptosis is genetically programmed, and recent work has disclosed several mRNA species and genes that are associated with apoptosis in specific tissues [5, 6].

Recent studies have demonstrated that apoptosis occurs in the ovary of several animal species. Most studies suggest that apoptosis is associated with follicular atresia. In ovarian physiology, follicular atresia is a key phenomenon through which the ovary eliminates those follicles that will not ovulate. The process of follicular selection results in the formation of one or more dominant follicles that will ovulate and in the concomitant atresia of multiple follicles that will eventually die. Despite the widespread occurrence and fundamental importance of atresia, its molecular mechanisms are still largely unknown. Hughes and Gorospe [7] have shown that eCG-induced atresia in the rat ovary is associated with apoptotic DNA fragmentation. Tilly and coauthors have demonstrated the presence of a ladder pattern in avian and porcine atretic follicles [8]. They have also shown that apoptosis occurs in rat granulosa cells in culture and that it can be prevented by the addition of certain specific growth factors [9]. All these studies strongly suggested that apoptosis occurs during follicular atresia. However, all were based on the analysis of DNA from either tissue homogenates or pooled cells and did not allow precise identification of the cell type(s) involved in apoptosis in the ovary. More recently, Billig et al. used both biochemical and in situ techniques to study apoptosis in the hypophysectomized, diethylstilbestrol (DES)-treated immature rat. DES withdrawal induced DNA fragmentation in granulosa cells of antral and preantral follicles. This was prevented by estrogen replacement, and restoration was achieved with androgen treatment [10]. In the same hypophysectomized, DES-treated rat model, treatment with a gonadotropin-releasing hormone agonist also induced DNA fragmentation in granulosa cells of preantral and antral follicles, whereas FSH decreased apoptosis.

The purpose of the present analysis was to study the pattern of apoptosis in ovarian follicles of untreated prepubertal and adult rats in order to correlate apoptosis with ovarian physiology. To achieve this objective, we combined the widely used technique of DNA analysis by gel electrophoresis with an in situ method [12]; together these meth-

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ods allow the identification of DNA fragmentation on conventional histologic sections while preserving tissue architecture. In order to further evaluate apoptosis during follicular atresia, we also studied eCG-treated rats 5 days after injection (in which atresia was induced by eCG withdrawal) and compared them to eCG-treated rats 48 h after injection (in which a cohort of healthy preovulatory follicles was induced by eCG). In all of our models, apoptosis specifically occured in atretic follicles as opposed to developing follicles. It occured mainly in granulosa cells, but scattered thecal cells were also involved. These findings support the proposition that apoptosis may be the mechanism of cell death during follicular atresia.

MATERIALS AND METHODS

Tissue Selection and Preparation for the In Situ Experiments

Female Sprague-Dawley rats (Taconic, Germantown, NY) were housed in well-ventilated rooms at 22°C with a 14L:10D cycle. All rats were fed Purina rodent laboratory chow (Ralston-Purina Co., St. Louis, MO) and given tap water ad libitum. All animals were killed using CO_2. Animals were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council. Ovaries were obtained from the following animal groups: 1) untreated prepubertal 26-day-old Sprague-Dawley rats (n = 5); 2) 26-day-old rats treated s.c. with 10 IU of eCG (Sigma Chemical Co., St. Louis, MO) and killed 2 days after the eCG administration (n = 2); 3) 26-day-old rats treated s.c. with 10 IU of eCG and killed 5 days after eCG (n = 2); and 4) adult, 60-day-old rats, having regular 4-day estrous cycles as determined by daily vaginal smears, killed on each day of the cycle (n = 2 per cycle day). Specimens were fixed in 4% buffered formaldehyde, dehydrated, and embedded in paraffin. Serial 4–6-μm sections were cut, mounted on gelatin-coated glass slides, and placed in a 60°C oven for 30 min. The sections were then deparaffinized in xylene and rehydrated in graded alcohol. Two sections from each ovary were stained with hematoxylin-eosin and evaluated for histologic identification of ovarian compartments.

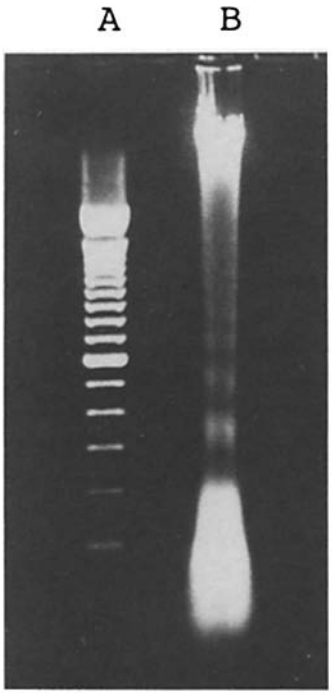


FIG. 1. Analysis of total cellular DNA obtained from immature rat granulosa cells (lane B) by 1.5% agarose gel electrophoresis with ethidium bromide staining. Fourteen micrograms of DNA was loaded. Lane A is a 100-bp ladder that was used as a marker.

Histologic Criteria for Atresia

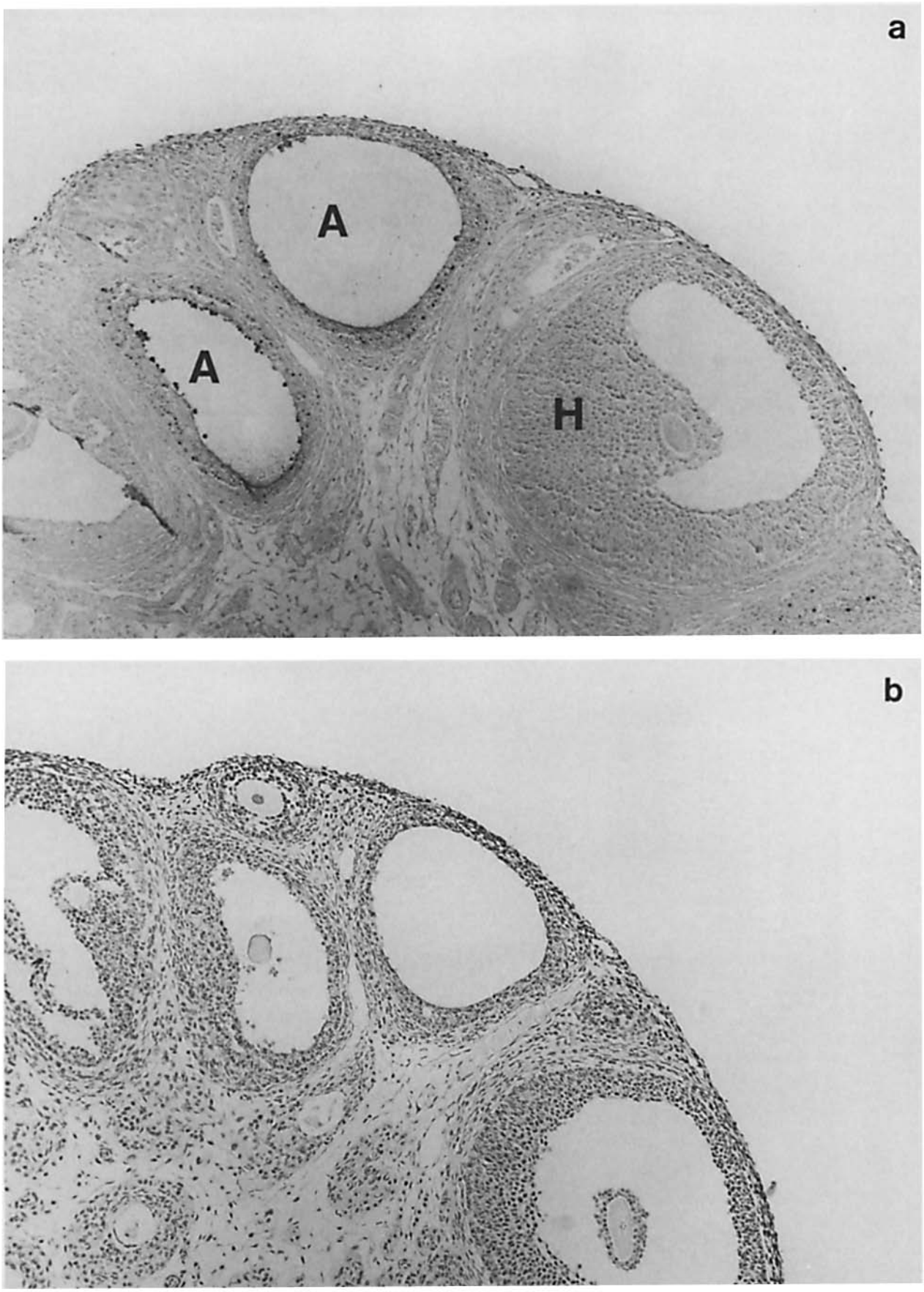
Atretic follicles were recognized by several histologic characteristics: disruption, pyknosis, and thinning of the granulosa cell layer with some degree of hypertrophy of the theca cell layer.

Detection of Apoptosis In Situ

Tissue sections were first incubated with proteinase K (20 μg/ml) for 15 min at room temperature (RT) to expose the 3′-hydroxy (3′-OH) ends of DNA, washed in double-distilled water (DDW), and then treated with 2% H_2O_2 in methanol for 10 min at RT to block endogenous peroxidase activity. The slides were then pretreated with terminal deoxynucleotidyl transferase (TDT) buffer (USB, Cleveland, OH) for 5 min at RT before incubation with biotinylated deoxyuridine triphosphate (dUTP; 20 μM, Biotin-21-UTP; Clontech, Palo Alto, CA) and TDT (0.3 eu/μl; USB) in TDT buffer for 1 h at 37°C in a humidified chamber. To stop the reaction, the slides were placed in double-strength SSC buffer (salt sodium citrate buffer) for 15 min at RT. After washing for 10 min at RT in PBS with 2% BSA, and then with PBS for 5 min at RT, slides were incubated with extra-avidin peroxidase (1:30; Sigma Chemical Co.) for 30 min at 37°C. The slides were washed again in PBS and then incubated in a solution of 3,3′-diaminobenzidine (0.1% in PBS) and 0.002% hydrogen peroxide for 2–3 min. They were then rinsed in water, dehydrated in graded alcohols, cleared in xylene, and mounted.

Controls

Positive and negative controls for the technique were included in each experimental run. To obtain positive controls, tissues were treated with DNase I (1–8 μg/ml; Boehringer-Mannheim Biochemicals, Indianapolis, IN) in DNase buffer for 10 min at RT before exposure to biotinylated dUTP and TDT. For negative controls, the ovarian sections were



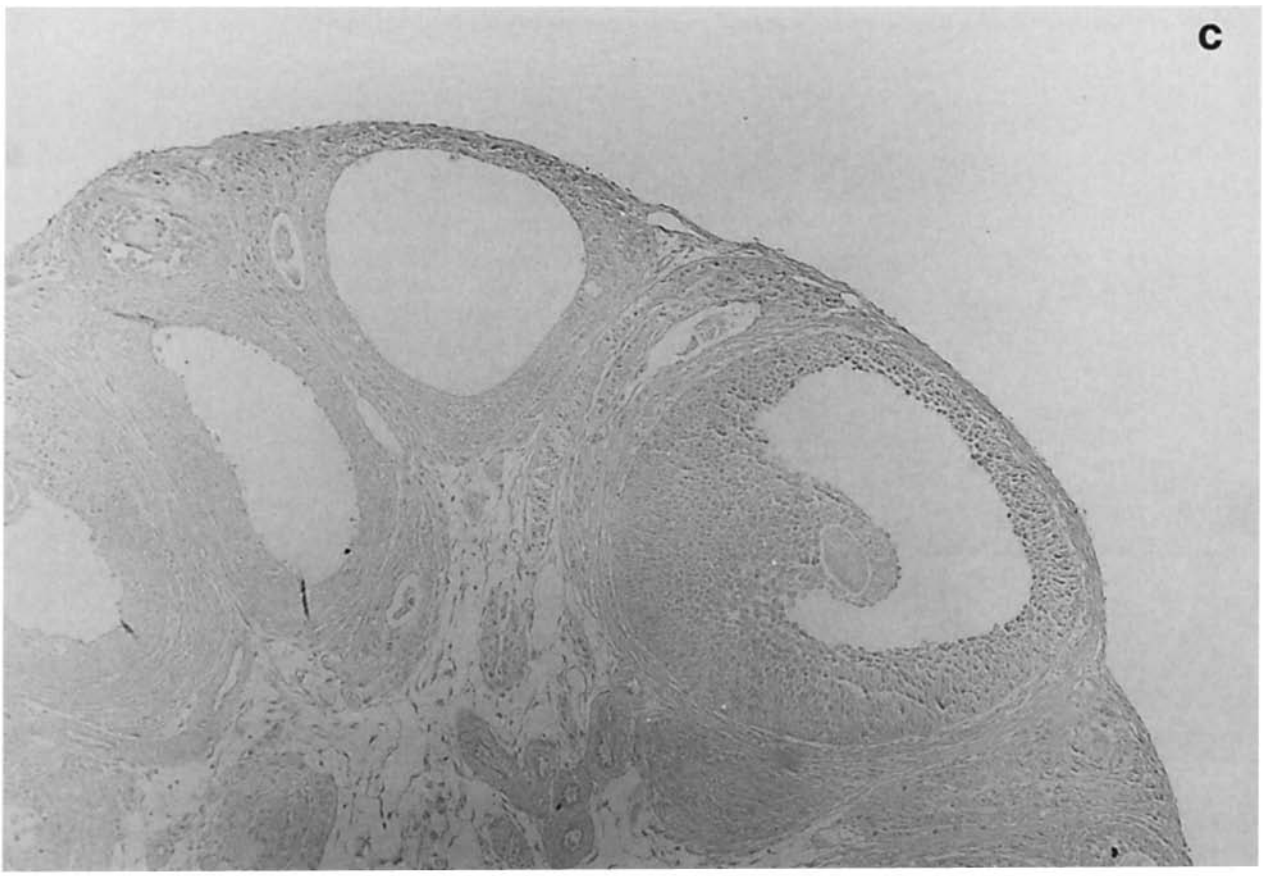


FIG. 2. Opposite and above. a) Staining of atretic follicles (A) in an immature rat ovary. Note the developing, healthy follicle (H) on the right, which is completely negative. ×100. b) Same ovary, positive control. ×100. c) Same ovary, negative control obtained by omitting the enzyme TDT. ×100.

incubated by omitting either the enzyme TDT or the substrate, biotinylated dUTP.

Animal Selection and Granulosa Cell Preparation for DNA Extraction

Twenty-six-day-old rats (n = 30) were killed by CO_2. Ovaries were quickly removed, placed in ice-cold McCoy's medium, and gently pierced about 30 times with sterile #00 insect needles. Ovaries were placed in nonenzymatic cell dissociation solution (Sigma Chemical Co.) at 37°C for 10 min and then incubated in hypertonic sucrose in McCoy's medium at RT for 5 min. To harvest the granulosa cells, the ovaries were placed in a 15-ml polypropylene test tube containing 1 ml of ice-cold McCoy's medium with gentamycin and 1% BSA (fraction V, Sigma Chemical Co.); they were gently pressed with a teflon pestle before filtering through a 50-μm nylon mesh. Cells were then centrifuged at 4°C at 1000–1500 rpm (200–500 × *g*) for 3–4 min and the pellet was resuspended in 5 ml of McCoy's medium. Cells were counted and viability was determined by the erythrosin B exclusion method. This cell suspension was then centrifuged again at 4°C, and the pellet was washed in PBS and stored at −70°C until processed for DNA extraction. The yield was 22 million cells per 30 rats, with a 36% viability.

DNA Extraction

The granulosa cell pellet was resuspended in 50 μl of ice-cold PBS; 350 μl of 6 M sodium iodide was added and gently mixed with the cell suspension. After phenol-chloroform extraction, the DNA was incubated with DNase-free RNase A (10 μg/ml, Boehringer-Mannheim) for 30 min at 37°C. This was again followed by phenol-chloroform extraction and ethanol precipitation. The DNA was resuspended in TRIS-EDTA buffer, and the DNA concentration was measured by spectrophotometry.

DNA Analysis

The DNA was analyzed by 1.5% agarose gel electrophoresis with ethidium bromide staining; 10 to 30 μg of DNA was loaded in each lane. A 100-bp ladder (GIBCO BRL, Gaithersburg, MD) was used as a marker.

RESULTS

The DNA extracted from immature rat granulosa cells displayed a characteristic ladder pattern on agarose gel

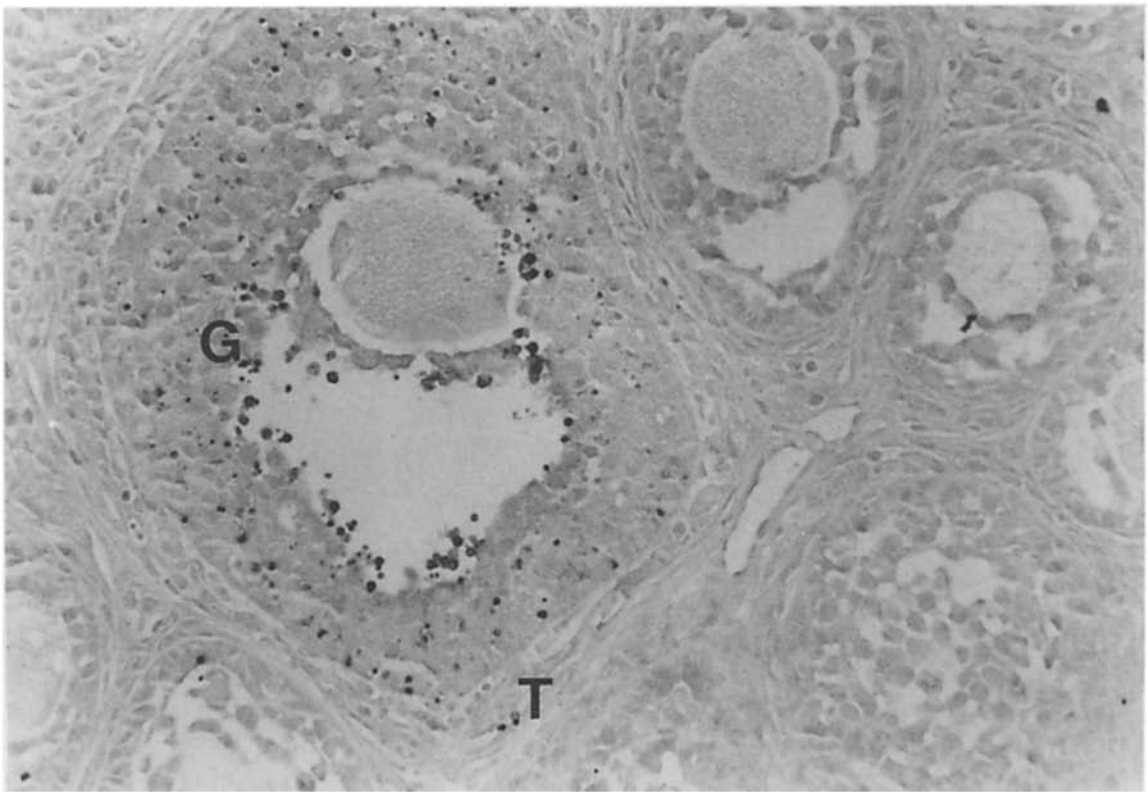


FIG. 3. High-power view of an atretic follicle in an immature rat ovary, demonstrating staining of scattered theca cells (T) as well as granulosa cells (G). ×200.

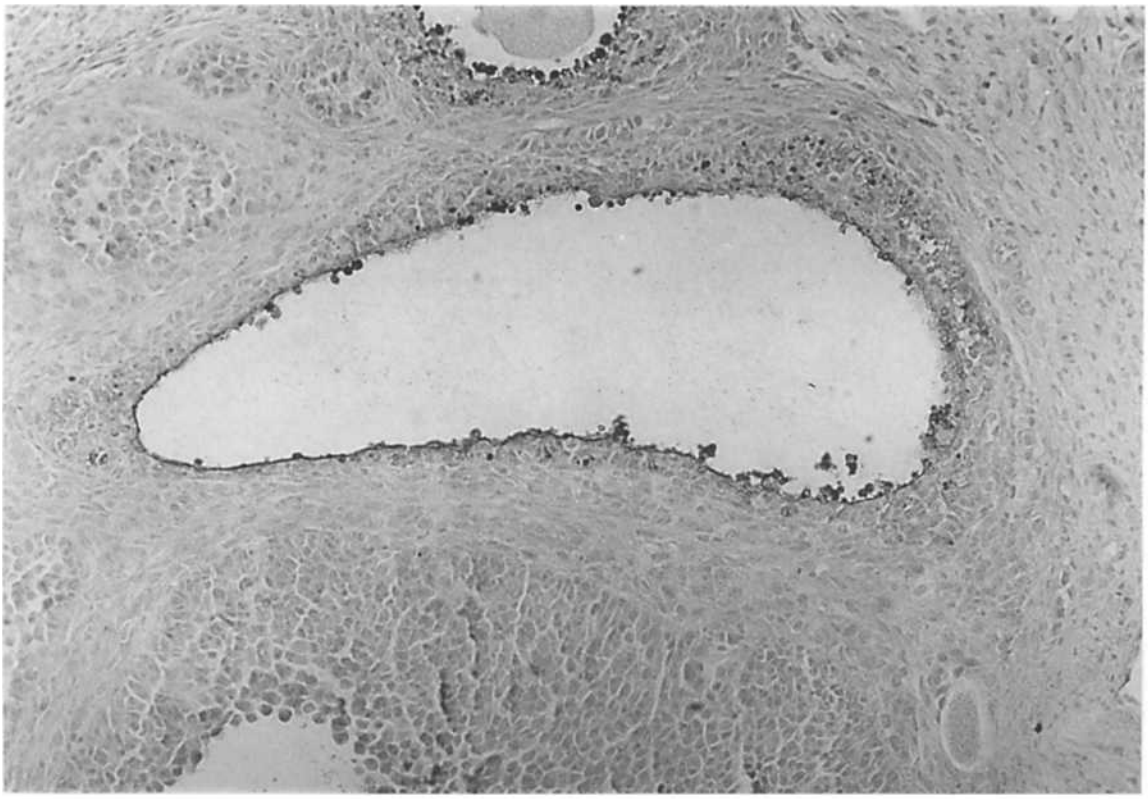


FIG. 4. Staining of an atretic follicle in an eCG-stimulated ovary, 48 h after the treatment. ×200.

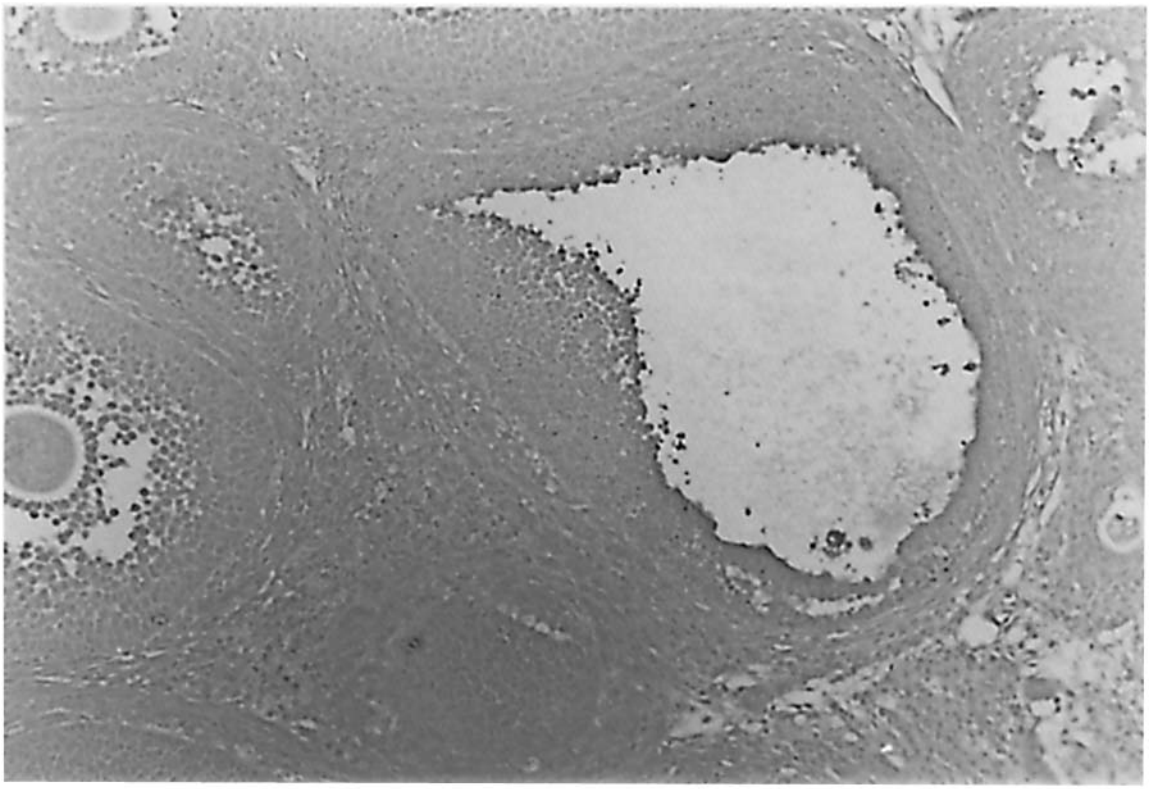


FIG. 5. Staining of atretic follicles in an eCG-stimulated ovary, 5 days after the treatment. ×200. Note the presence of stained apoptotic nuclei in several adjacent follicles.

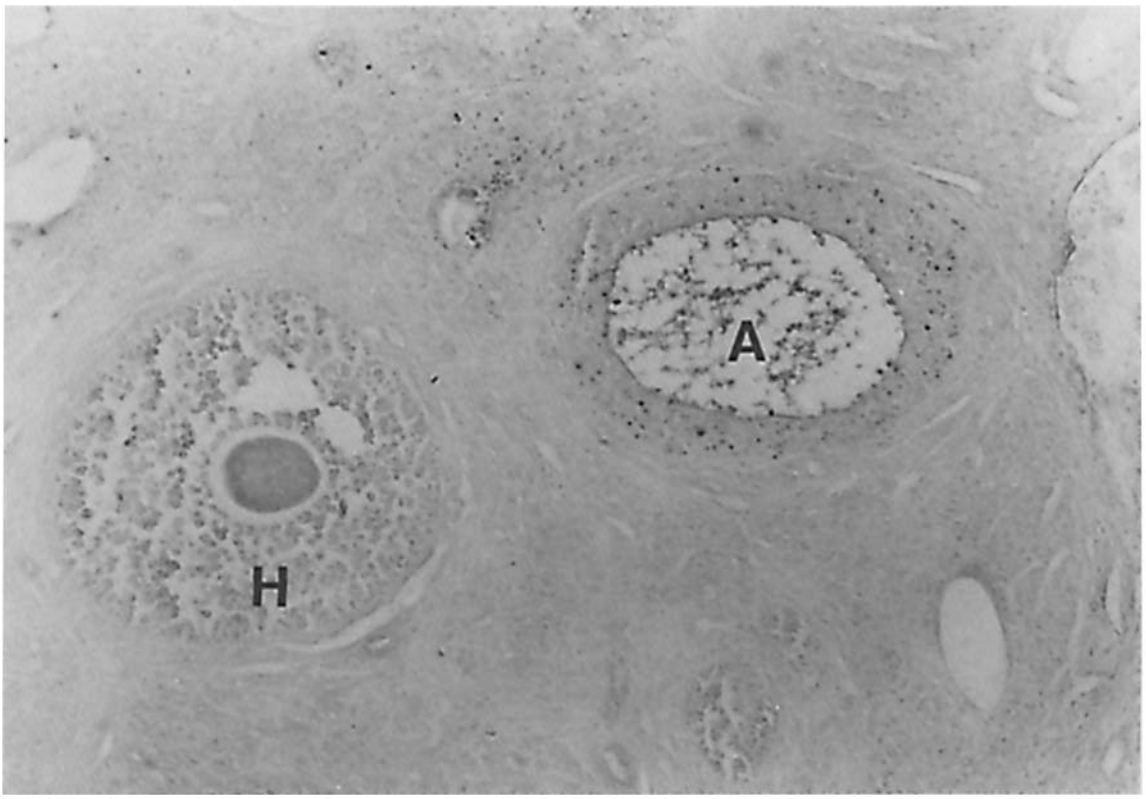


FIG. 6. Staining of an atretic follicle (A) in an adult rat ovary. Note the healthy follicle (H) on the left, which is negative. ×100.

electrophoresis (Fig. 1), indicating the presence of apoptotic cells. Ovaries from immature rats of the same age as those used in the in situ experiments displayed selective staining of the nuclei of atretic follicles. This pattern was present in both preantral and antral atretic follicles (Fig. 2a). Healthy preantral or antral follicles were consistently 0negative (Fig. 2a). Positive controls in all cases displayed uniform staining of all nuclei (Fig. 2b). Omission of either the terminal transferase or the biotinylated dUTP consistently led to completely negative results (Fig. 2c).

Although the nuclear staining involved mainly granulosa cells in such follicles, scattered cells in the theca interna layer were also involved (Fig. 3).

Rat ovaries stimulated with eCG contained scattered positive atretic follicles 48 h after the eCG injection (Fig. 4). Five days after the eCG injection, most of the follicles were stained (Fig. 5).

Adult rat ovaries also displayed staining of atretic follicles throughout the cycle (Fig. 6). As in the ovaries from the immature rats, the cells involved were mainly granulosa cells, but theca cells were occasionally stained.

DISCUSSION

The hallmark of apoptosis is the presence of a typical internucleosomal DNA fragmentation that is identified by the appearance of a ladder pattern on gel electrophoresis [2]. Recognition of the ladder pattern, however, requires relatively large amounts of DNA (15–30 μg for an agarose gel with ethidium bromide staining and 15 ng for gels run after 3′-end labeling of DNA with P32-ddATP) [13]. In addition, the technique is performed with pooled DNA extracted from tissue or cell homogenates, and this does not allow identification of the specific tissue compartments (i.e., preantral vs. antral follicles, healthy vs. atretic follicles) or cell types (i.e., granulosa vs. theca cells) involved in apoptosis. The advantages offered by the present technique are the possibility of detecting apoptosis in situ on conventional histologic sections without disruption of the tissue morphology and the possibility of visualizing DNA fragmentation at the single cell level.

Our method of harvesting granulosa cells from prepubertal rat ovaries, described by Anderson and coauthors [14, 15], provides a granulosa cell preparation that is 95% pure. The presence of a ladder pattern in DNA from freshly harvested granulosa cells indicated the presence of apoptotic granulosa cells in the untreated immature rat ovary, in accordance with previous reports [7, 9]. Although atretic follicles are the most likely site for these apoptotic cells, this assumption needed to be confirmed. Moreover, the possibility that cell types other than granulosa cells were involved in apoptosis could not be explored by the conventional ladder method. The present technique, which allows the identification of apoptosis in situ with preservation of the tissue architecture, is well suited for these purposes.

The present study provides evidence that apoptosis in the rat ovary is associated with atretic follicles. The cells involved are for the most part granulosa cells; however, occasional theca cells also undergo apoptosis. This is consistent with the fact that during the atretic process granulosa cells become pyknotic and die whereas most of the theca cells are reincorporated in the ovarian interstitium [16, 17]. Both preantral and antral atretic follicles displayed stained nuclei: these tended to be located in higher numbers in the layers of granulosa cells that are closer to follicular fluid. However, no specific pattern was recognized consistently, and some follicles also displayed some thecal staining. These differences may be dependent on the fact that different follicles are in different stages of atresia and the fact that apoptosis is a transitory phenomenon. In general, examination of multiple sections revealed that most of the atretic follicles, if not all, both preantral and antral, had at least some apoptotic cells.

The eCG-stimulated rat model was chosen for two reasons. First, 48 h after the eCG injection the immature rat ovary displays a relatively uniform population of preovulatory follicles. DNA from fresh granulosa cells obtained from these rats does not display a ladder pattern on gel electrophoresis [7, 9]; but the in situ technique shows some apoptotic cells in scattered atretic follicles, demonstrating that this method is extremely sensitive. Second, 5 days after eCG, most of the follicles are atretic because of withdrawal of the hormonal stimulus. DNA from fresh granulosa cells obtained from these rats displays apoptosis on gel electrophoresis [7].

Technically, this method is simple and reproducible. Positive controls, in which the DNA is cut by DNase I, represent evidence that the DNA fragments are uniformly accessible to the terminal transferase—i.e., that the proteolytic treatment has been effective—and must be included in all experiments. Negative controls rule out the presence of nonspecific staining. Particularly important are the controls obtained by incubating the slides with the biotinylated substrate in the absence of the enzyme, since biotin is known to produce nonspecific artifactual staining when employed in immunohistochemistry. Again, negative controls need to be included in all experiments to make it possible to discriminate between background and real positive staining.

In conclusion, this study demonstrates that apoptosis occurs in atretic follicles in the normal rat ovary, indicating that apoptosis may be the mechanism of follicular atresia in physiologic conditions. Staining of the atretic follicles, but not the healthy follicles, was a consistent finding in all of the animal models examined.

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