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Apoptotic and proliferative changes during induced atresia of pre-ovulatory follicles in the rat

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Atresia, a degenerative process through which many follicles are removed from the growing pool, involves apoptotic changes in the follicular granulosa cells. To identify histochemical markers of early stages of atresia, an in-vivo rat model was used which allowed the study of atresia of pre-ovulatory follicles in a synchronized and chronological order. By blocking the pre-ovulatory luteinizing hormone surge with a gonadotrophin-releasing hormone (GnRH) antagonist, ovulation of the pre-ovulatory follicles is prevented, after which these follicles became atretic. The first morphological sign of atresia (pyknotic granulosa cell nuclei) was found 27 h after injection of GnRH antagonist. Since the pre-ovulatory follicles gradually become atretic in a synchronous fashion, this model provided an opportunity to study and define markers of future atresia in pre-ovulatory follicles. Atresia involves apoptosis of granulosa cells, and therefore internucleosomal DNA fragmentation was examined. Using the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labelling (TUNEL) assay it was found that the first sign of internucleosomal DNA fragmentation in granulosa cells of pre-ovulatory follicles was detectable 24 h after GnRH antagonist treatment. In order to find an upstream marker of atresia, the 5-bromo-deoxyuridine (BrdU) labelling index was used as a measure of proliferation. Already at 14 h after GnRH antagonist treatment, when morphological signs of atresia were not yet present, a clear decrease in BrdU labelling index was found in the granulosa cells.

Key words: atresia/5-bromo-deoxyuridine/DNA fragmentation/ovary

Introduction

During female reproductive life, most ovarian follicles at some stage of their development undergo a degenerative process called atresia, and only few follicles reach the ovulatory stage (Byskov, 1978). The largest part of atresia takes place during the late pre-antral to early antral stages, when continued growth is dependent upon gonadotrophins and a selection is made of follicles that grow on until the pre-ovulatory stage. Several atretogenic and anti-atretogenic factors have been identified. Gonadotrophins are survival factors that prevent atresia (Braw and Tsafriri, 1980; Uilenbroek *et al*., 1980; Chun *et al*., 1994), as are the growth factors epidermal growth factor, transforming growth factor-α), basic fibroblast growth factor (Tilly *et al*., 1992), insulin-like growth factor-I (Chun *et al*., 1994), and the cytokine interleukin (IL)-1β (Chun *et al*., 1995). Also oestrogens (Billig *et al*., 1993) and inhibin (Woodruff *et al*., 1990) have been shown to suppress follicular atresia, whereas androgens (Billig *et al*., 1993), activin (Woodruff *et al*., 1990), IL-6 (Gorospe and Spangelo, 1993), tumour necrosis factor-α) (Kaipia *et al*., 1996), Fas-ligand (Quirk *et al*., 1995), and gonadotrophin-releasing hormone (GnRH) (Billig *et al*., 1994) exert atretogenic actions.

Studies have shown that follicular atresia is an apoptosis-mediated process (Tsafriri and Braw, 1984). Apoptosis is a process of active and selective cell deletion that requires active gene expression. It is characterized by certain morphological phenomena such as pyknosis of cell nuclei and the formation of apoptotic bodies (Kerr *et al*., 1972). The morphological changes that occur in granulosa cells of follicles undergoing atresia match all apoptosis-related morphological characteristics (Tsafriri and Braw, 1984). In addition, internucleosomal DNA fragmentation, which is a hallmark of cells undergoing apoptosis, occurs in atretic follicles (Tilly and Hsueh, 1993; Tilly, 1996).

Despite numerous studies on atresia, the exact mechanisms that determine whether a follicle will continue to grow or undergo atresia are still unknown. The main purpose of this study was to characterize an animal model, which can be used to find markers for atresia which become apparent before any morphological sign of atresia is detectable. An in-vivo model was used, based on the prevention of the pre-ovulatory LH with a GnRH antagonist, which allowed the study of synchronized atresia of pre-ovulatory follicles in adult rats (Uilenbroek *et al*., 1998). In this model, all pre-ovulatory follicles that are healthy at pro-oestrus are committed to become atretic. Although under normal physiological conditions the major peak of atresia occurs when developing follicles are in the small antral follicle stage, it is discussed whether a marker for future atresia of pre-ovulatory follicles can be used to identify the early stages of atresia at earlier stages of follicle development. In the present model, the proliferative activity of granulosa cells investigated using 5-bromodeoxyuridine (BrdU) labelling appeared to be a promising upstream marker of early atresia.

Materials and methods

Animals and tissue preparations

The experiments were performed using 3–4 months old Wistar rats (RP-1 strain). The animals were locally bred and were maintained under controlled conditions (20–23°C, lights on from 05.00 to 19.00). Animal care was performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Vaginal smears were taken daily at about 09.00. Only animals displaying at least two consecutive 5 day reproductive cycles prior to the experiment were used.

The LH urge was blocked by an s.c. injection of GnRH antagonist (20 μg; Org 30276; Organon NV, Oss, The Netherlands) at 10.00 of pro-oestrus. To prevent the occurrence of an LH surge the next day, a second injection of GnRH antagonist (20 μg) was given at 10.00 the following day. Animals were killed by CO_2 inhalation at 0, 7, 14, 24, 27, 31, 48, 72 and 96 h after the first injection of GnRH antagonist.

Ovaries were dissected out and fixed in buffered 4% (v/v) formaldehyde (pH 6.9; 16–24 h at room temperature) for histology, immunohistochemistry and in-situ DNA labelling. The fixed ovaries were embedded in paraffin wax and 10 μm sections were mounted on slides and stained with haematoxylin and eosin. Sections used for immunohistochemistry or in-situ DNA labelling were mounted on slides coated with 3-amino-isopropyl- triethoxysilane (APES; Sigma, St Louis, MO, USA).

Morphological evaluation of atresia

The morphology of the pre-ovulatory follicles was examined in ovaries of three animals per single time-point. Both ovaries of each animal were used. Every fifth section of each ovary was mounted on a slide to ensure that all pre-ovulatory follicles were examined. Only sections in which the nucleus of the oocyte was visible were used for histological examination, to guarantee that the pre-ovulatory follicles were examined only once. Based on light-microscopical observation, pre-ovulatory follicles were divided into two stages of atresia (Osman, 1985). In stage I, degenerative changes are only present in the granulosa layer; in stage II, changes are also present in the oocyte which shows signs of resumption of meiosis, e.g. formation of a pseudo-maturation spindle. Follicles in stage I can be further subdivided into stage Ia and stage Ib. At stage Ia an overall shrinkage of the granulosa wall is apparent at a low magnification and detailed degenerative changes, such as pyknosis of granulosa cell nuclei, are seen at a higher magnification in scattered small areas (Figure 1C and D). At stage Ib many pyknotic cells are found throughout the entire granulosa layer. Many nuclear fragments (apoptotic bodies) are seen at the periphery of the antrum (Figure 1E and F). Stage II can also be subdivided into stage IIa, in which the degenerating oocyte is still surrounded by an envelope of degenerating cumulus cells (Figure 1G and H), and stage IIb, in which the oocyte lies almost solitary in the antrum without any surrounding cumulus cells. No or only a few nuclear particles are seen in the antrum, but macrophages are usually present (Figure 1I and J).

During the course of atresia the thickness of the granulosa layer is reduced dramatically. This reduction starts at any place of the layer except at the cumulus cells and can be first seen during late stage Ia. During stage II the complete granulosa layer is affected.

In-situ DNA labelling analysis by TUNEL assay

In-situ DNA labelling analysis was performed in sections of the same ovaries and on the same pre-ovulatory follicles used in the histological examination of the pre-ovulatory follicles. Approximately 36 pre-ovulatory follicles were examined per time-point. Tissue sections were rehydrated and incubated with proteinase K (7.5 μg/ml, Boehringer Mannheim, Mannheim, Germany) in phosphate-buffered saline (PBS; Merck, Hohenbrunn, Germany, 0.15 mol/l NaCl, 0.03 mol/l KCl, 0.01 mol/l $Na_2HPO_4.2H_2O$, 0.01 mol/l KH_2PO_4, pH 7.5) for 15 min at room temperature to expose the 3′-hydroxy ends of DNA. Subsequently, the sections were rinsed in distilled water and treated with 2% H_2O_2 in PBS for 5 min at room temperature to block endogenous peroxidase activity. The sections were pretreated with terminal deoxynucleotidyl transferase (TDT) buffer [0.1 mol/l sodium cacodylate, 1 mmol/l cobalt chloride, 1 mmol/l dithiothreitol (DTT), 5% bovine serum slbumin (BSA)] for 5 min before incubation with biotinylated deoxyuridine triphosphate (1:100; biotin-dUTP labelling mix: final concentration: 10 μmol/l each dATP, dGTP, dCTP and 6.5 μmol/l dUTP, 3.5 μmol/l biotin-16-dUTP; Boehringer Mannheim) and TDT (final concentration: 0.3 U/μl; Promega, Madison, WI, USA) in TDT buffer (Merck) for 2 h at 37°C in a humidified chamber. As a negative control, one slide was incubated with the same reaction mixture excluding TDT. To stop the reaction the slides were transferred into TB buffer (300 mmol/l NaCl, 30 mmol/l sodium citrate, pH 8.0, Merck) for 15 min. After rinsing for 10 min in PBS containing 2% BSA and subsequently for 5 min in PBS, the slides were incubated with streptavidin-biotin-peroxidase complex (1:200; DAKO, Glostrup, Denmark) for 30 min. Peroxidase activity was developed with 3,3′-diaminobenzidine (DAB; 0.75 mg/ml; Fluka, Buchs, Switzerland) in PBS for 5 min. Finally, the sections were rinsed in tap water, counter-stained with haematoxylin, dehydrated in graded alcohols, cleared in xylene and mounted.

Immunohistochemistry of BrdU

For BrdU immunostaining, two animals per time-point were used. These animals were different from the animals used in the morphology and TUNEL study. The animals were first treated with GnRH antagonist and received a single i.p. injection with BrdU (Boehringer Mannheim) dissolved in saline (100 mg/kg body weight) 2 h before the animals were killed. Of each rat the BrdU labelling index was determined in four pre-ovulatory follicles, which were chosen at random.

For BrdU immunostaining, sections were quenched in 3% H_2O_2 for 12 min, washed with PBS and incubated with 0.1% pronase (Boehringer Mannheim) in distilled water at 37°C for 10 min. After rinsing in PBS at 4°C the sections were treated with 2 N HCl for 30 min at 37°C, and neutralized with 0.1 mol/l borate buffer (0.1 mol/l borax, 0.1 mol/l boric acid, pH 8.5, Sigma). After rinsing in PBS, sections were subsequently incubated with anti-BrdU (1:25; Sigma) for 30 min at room temperature and subsequently peroxidase-conjugated goat anti-mouse antibody (1:100; Sigma) in PBS containing 4% normal goat serum (Sigma) for 30 min at 37°C. Immunoreactive sites were visualized with DAB. Sections were counter-stained with haematoxylin.

BrdU labelling index of the granulosa cells was determined for an area opposite the oocyte and for an area adjacent to the oocyte (Figure 4B). The labelling index of a follicle is represented by the number of labelled granulosa cells expressed as a percentage of all granulosa cells counted in the designated areas. Cells were counted with the aid of an eyepiece with a grid.

Statistical analysis

Results are expressed as means ± SEM. The data were evaluated for statistically significant differences by one-way analysis of variance (ANOVA) followed by a Duncan multiple comparison test using Statistics Package for Social Sciences 7.5 (SPSS Inc., Chicago, IL, USA) computer software. $P < 0.05$ was considered to be significant.

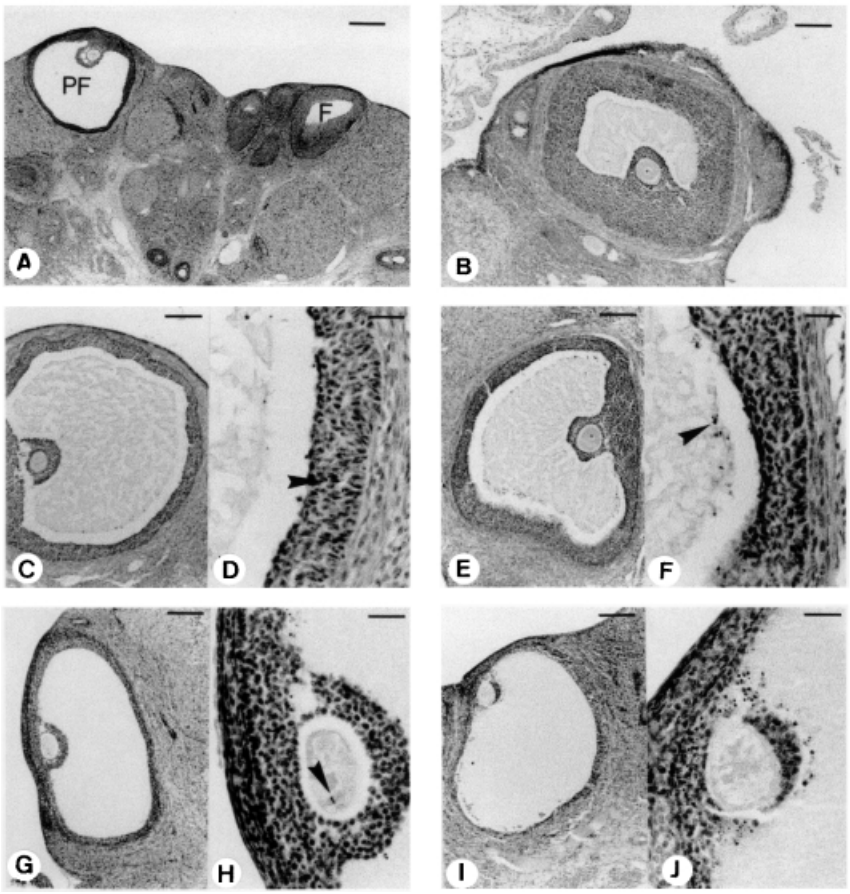

Figure 1. Histology of pre-ovulatory follicles at different stages of atresia. (**A**) Ovarian histology 3 days after blocking ovulation with gonadotrophin-releasing hormone antagonist (P+72). The ovaries showed pre-ovulatory follicles in advanced stages of atresia (PF). Another set of antral follicles (F) was present, which would ovulate between P+96 and P+120. (**B**) Healthy pre-ovulatory follicle. (**C**, **D**) Stage Ia of atresia. Note the presence of pyknotic granulosa cells in scattered small areas of the granulosa layer (arrowhead). (**E**, **F**) Stage Ib of atresia. The complete granulosa layer was affected by degeneration. Note apoptotic bodies at the periphery of the antrum (arrowhead). (**G**, **H**) Stage IIa of atresia. The granulosa layer was thin, and in the oocyte, which was still surrounded by cumulus cells, a pseudo-maturation spindle was visible (arrowhead). (**I**, **J**) Stage IIb of atresia. The oocyte lay almost solitary in the antrum, while many macrophages were present in the antrum. **D**, **F**, **H**, **J** are higher magnifications of a part of the pre-ovulatory follicles in **C**, **E**, **G** and **I** respectively. **A**: scale bar = 250 μm; **B**, **C**, **E**, **G**, **I**: scale bar = 100 μm; **D**, **F**, **H**, **J**: scale bar = 50 μm.

Results

Morphology of pre-ovulatory follicles after GnRH antagonist treatment

To investigate whether the pre-ovulatory follicles gradually become atretic in a synchronous fashion after GnRH antagonist treatment, the pre-ovulatory follicles were examined at several time-points after the first injection with GnRH antagonist.

Two successive injections of GnRH antagonist, one at pro-oestrus and one 24 h later, resulted in blockade of ovulation in all rats studied, as ovarian histology revealed large unruptured follicles and no recent corpora lutea. Ovaries contained follicles of all developmental stages and corpora lutea from previous cycles. At the time-point 72 h after the first GnRH antagonist injection (P+72), a new cohort of antral follicles can be distinguished which will ovulate between P+96 and P+120 (Figure 1A). The non-ovulated pre-ovulatory follicles at various time-points after GnRH antagonist injection were examined, and classified as non-atretic or as being in one of the four stages of atresia (Figure 1B-J; Table I). Except in a single pre-ovulatory follicle at P+14, the first pyknotic granulosa cells were found 27 h after the first injection with GnRH antagonist (P+27). Pyknotic granulosa cells were found in all pre-ovulatory follicles at P+48, while at P+96 in addition to the pyknotic granulosa cells, all oocytes showed signs of resumption of meiosis (Figure 1G and H). The last of the granulosa cells to become pyknotic were the cumulus cells. The granulosa wall of the pre-ovulatory follicles became thinner at the end of stage Ia. At stage IIb the thickness of this wall was reduced to 2–3 layers of granulosa cells, while in the antrum many macrophages were found (Figure 1I and J).

The data given in Table I clearly demonstrate that the number of morphologically atretic pre-ovulatory follicles becomes larger as time passes following the first GnRH antagonist injection.

In-situ 3′ end labelling of DNA fragments

One of the hallmarks of apoptotic cell death is internucleosomal DNA fragmentation. The TUNEL-assay was used to detect 3′-hydroxy DNA fragments in the pre-ovulatory follicles undergoing synchronized atresia.

Table I. Atresia of pre-ovulatory follicles after blocking ovulation by gonadotrophin-releasing hormone (GnRH) antagonist on the morning of pro-oestrus

Hours after GnRH antagonist treatment	Total no. of pre-ovulatory follicles in three rats	No. of non-atretic pre-ovulatory follicles per rat[a]	No. of atretic pre-ovulatory follicles per rat			
			Early atretic		Late atretic	
			IA[a]	Ib[a]	IIa[a]	IIb[a]
0	34	11.3 ± 1.3	–	–	–	–
7	38	12.6 ± 0.3	–	–	–	–
14	38	12.7 ± 0.9	0.3 ± 0.3	–	–	–
24	38	12.7 ± 0.3	–	–	–	–
27	37	10.7 ± 0.3	1.3 ± 0.3	0.3 ± 0.3	–	–
31	36	6.7 ± 0.9	3.3 ± 0.7	2.0 ± 1.0	–	–
48	31	–	3.7 ± 1.2	6.7 ± 1.3	–	–
72	39	–	–	9.3 ± 0.9	3.7 ± 0.3	–
96	33	–	–	–	10.0 ± 0.0	1.0 ± 0.6

[a]Values represent the mean of the total number of pre-ovulatory follicles per rat ± SEM.

Positive staining was found in granulosa cells and sometimes in the antrum of atretic follicles (Figure 2A). In atretic follicles with antral cavities, granulosa cells adjacent to the antrum were frequently more heavily labelled than mural granulosa cells (Figure 2D–F). Also apoptotic bodies and macrophages present in the antrum during the more advanced stages of atresia were stained (Figure 2F), as well as corpora lutea (Figure 2C). At time-point P+24 the first TUNEL-positive granulosa cells were found in pre-ovulatory follicles, although staining was faint and in some cases overshadowed by the background staining that was found to be somewhat variable between experiments (Figure 2A and B). Only at this time-point were TUNEL-positive granulosa cells seen in pre-ovulatory follicles that were healthy-looking according to morphological criteria. At all later time-points (P+27, P+31, P+48, P+72 and P+96) perfect overlap was found between the intense TUNEL staining and morphological atresia (Figure 2C–F). In general, the amount of background staining was low in the negative controls.

BrdU immunostaining and labelling index

Atresia of follicles may be accompanied by a decrease in granulosa cell proliferation. BrdU labelling of granulosa cells was used as a proliferation marker, since BrdU is a thymidine analogue that is incorporated into DNA during the S phase of the cell cycle (Gratzner, 1982).

Nuclear BrdU staining was found in granulosa and theca cells of follicles of all developmental classes, except for the primordial and the primary follicles. Some cells of corpora lutea also showed immunostaining, just as macrophages found in the interstitial tissue and in the antrum of follicles at more advanced stages of atresia. Background staining was very low and restricted to the corpora lutea and erythrocytes, probably caused by endogenous peroxidase activity. In the oocytes no staining was present (Figure 3A). In the pre-ovulatory follicles the outer granulosa cell layers showed a very low number of labelled cells (Figure 3B). The cumulus cells were the last granulosa cells to lose their BrdU immunoreactivity during atresia and BrdU incorporation into the nuclei of granulosa cells was absent during advanced stages of atresia (Figure 3D).

Figure 4 shows the BrdU labelling index of granulosa cells in the vicinity of the oocyte (cumulus) and granulosa cells more distant from the oocyte (non-cumulus) at the different time-points after the first GnRH antagonist injection, as a percentage of the BrdU labelling index of time-point 0 h (control). At almost all time-points, the labelling index of the granulosa cells found in the vicinity of the oocyte was higher than the other granulosa cells. The BrdU labelling index of the granulosa cells not located in the vicinity of the oocyte (Figure 3B) was significantly decreased at P+14, when no morphological signs of atresia were present. Table II shows for two rats the mean ± SEM of the BrdU labelling index of cumulus and non-cumulus granulosa cells of four pre-ovulatory follicles.

Discussion

There is a lack of histochemical markers signifying future atresia in ovarian follicles that do not yet show morphological characteristics of atresia. Until now, all markers that have been used identify follicles, which, on the basis of morphological characteristics, can be judged to be atretic. Thus, upstream markers for atresia can only be identified in a system in which it is known that all follicles of a certain class will go into atresia within a certain time span, and in a synchronized manner. In the present study such a system was used: pre-ovulatory follicles (all of the same class) at pro-oestrus were prevented from ovulating by administration of GnRH antagonist. Thereby a pool of recognizable follicles (all pre-ovulatory) was guaranteed to go into atresia in a synchronous manner. Examination of very early time-points in this system allowed the identification of changing histochemical markers before any signs of atresia could be seen on the basis of morphological criteria.

Thus, administration of GnRH antagonist to adult female rats, on the morning of pro-oestrus and 24 h later, resulted in prevention of ovulation after which the pre-ovulatory follicles became atretic. The second injection was needed to block the delayed LH surge that would normally occur 24 h after the first injection with GnRH antagonist. In a previous study

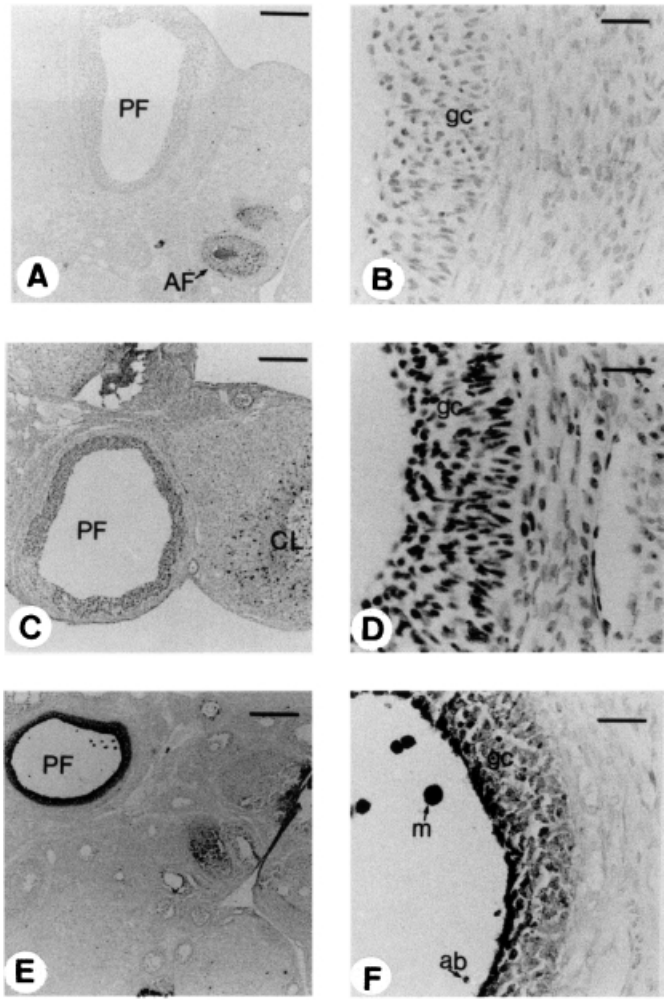

Figure 2. In-situ detection of fragmented DNA by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labelling assay. (**A**, **B**) Ovary at the morning of pro-oestrus. Positive staining was found in granulosa cells and antrum of atretic follicles (AF). No staining was found in the granulosa cells (gc) of the pre-ovulatory follicle (PF). (**C**, **D**) Ovary at P+24. Faint staining was found in the granulosa cells (gc) of the pre-ovulatory follicle (PF), whereas no pyknosis could be found. Corpora lutea (CL) were also positive. (**E**, **F**) Ovary at P+48. Intense staining was found in the granulosa cells (gc) and in apoptotic bodies (ab) and macrophages (m) in the antrum of the pre-ovulatory follicle (PF). **B**, **D** and **F** are higher magnifications of a part of the pre-ovulatory follicle in **A**, **C** and **E** respectively. **A**, **C**, **D**: scale bar = 250 μm; **B**, **D**, **F**: scale bar = 50 μm

the application of this model in the study of atresia of pre-ovulatory follicles was demonstrated (Uilenbroek *et al*., 1998). In the present study a more accurate timing of the process of atresia was needed to evaluate upstream markers of atresia and therefore more time-points were included than in the previous study. From the present study it appeared that the first morphological sign of atresia in the pre-ovulatory follicles, i.e. pyknosis of a few granulosa cell nuclei, was found 27 h after the first injection with GnRH antagonist. Twenty-one hours later (P+48) all pre-ovulatory follicles showed pyknosis of granulosa cell nuclei and the extent of the atretic process progressed with time. This model thus provides an excellent way to study future atresia. This model, however, has some limitations. Pre-ovulatory follicles under normal physiological conditions do not go into atresia. The majority of atresia under physiological conditions occurs in large pre-antral and small antral follicles (Kaipia and Hsueh, 1997). Since it appears that no animal models exist in which all large pre-antral and small antral follicles become atretic according to a predictable schedule, the model presented herein was used. There is no indication that the process of induced atresia in pre-ovulatory follicles as in the present model is different from the process of atresia in large pre-antral and small antral follicles. Indeed, the morphology of atretic large pre-antral and small antral follicles is not different from the morphology of atretic pre-ovulatory follicles, and it is assumed that the biochemical processes (and thus biochemical markers) underlying the morphological changes occurring during atresia in pre-ovulatory follicles may reflect changes occurring in large pre-antral and small antral follicles.

The model presented here has several advantages over earlier studies in which synchrony of atresia of pre-ovulatory follicles was studied after hypophysectomy of rats on the morning of pro-oestrus (Talbert *et al*., 1951; Braw *et al*., 1981), or after pentobarbital (Nembutal) treatment (Braw and Tsafriri, 1980; Uilenbroek *et al*., 1980). First of all, the rate with which the pre-ovulatory follicles go into atresia differs for the three different experimental treatments. In the present model the first sign of atresia was found 27 h after GnRH antagonist treatment. Following injection of pentobarbital, the first morphological signs of atresia are found after 72 h (Braw, 1980; Uilenbroek *et al*., 1980), whereas in hypophysectomized rats advanced stages of atresia are already found within 24 h (Talbert *et al*., 1951; Braw and Tsafriri, 1981). The differences in the rate of atresia in the three treatment groups may be explained by the extent of reduction of the basal level of LH. Serum LH concenrations 24 h after treatment with 20 μg or 100 μg GnRH antagonist on the morning of pro-oestrus were respectively 0.72 ± 0.01 and 0.20 ± 0.01 ng/ml ($P < 0.001$) (Uilenbroek *et al*., 1998). The rate of atresia increases with the dose of GnRH antagonist used, which is in keeping with the findings that in the case of hypophysectomy serum LH concenrations after 25 h are not detectable and the rate of the atretic process is high, whereas after pentobarbital treatment (37 mg/kg body weight) the serum LH concenration is 1.57 ± 0.13 ng/ml (Uilenbroek *et al*., 1998) and the rate of the atretic process low. The intermediate rate of atresia found in the GnRH antagonist treatment model allows convenient study of the process of atresia in the pre-ovulatory follicles. Furthermore, hypophysectomy is less specific, since by this treatment not only is the LH surge blocked, but the production of other hormones and growth factors is also influenced, which might lead to unwanted side-effects. Another advantage of the use of GnRH antagonist is that it is applicable in different doses by which delay or acceleration of the rate of atresia is possible (Uilenbroek *et al*., 1998). GnRH antagonist may have direct effects on the ovary itself, since GnRH can bind specifically to ovarian homogenates (Hsueh and Jones, 1981) and GnRH receptors have been found in the rat ovary (Kogo *et al*., 1999). However, there are no indications for direct effects of the GnRH antagonist on the ovary itself. Incubation

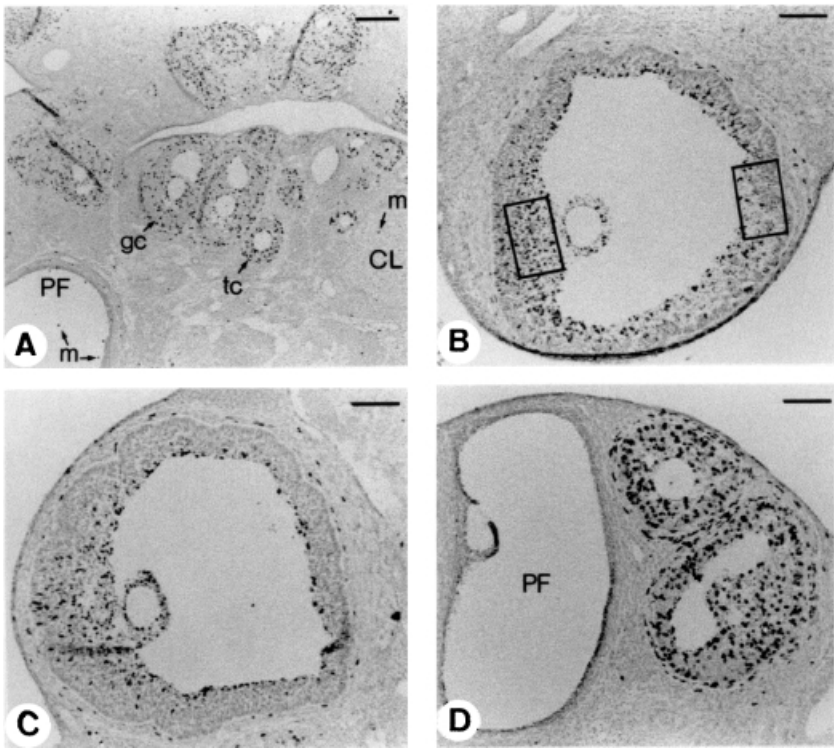

Figure 3. Immunohistochemical localization of 5-bromo-deoxyuridine (BrdU) in pre-ovulatory follicles after gonadotrophin-releasing hormone (GnRH) antagonist treatment. (**A**) Overview of ovary 72 h after GnRH antagonist treatment (P+72). Immunostaining was found in granulosa (gc) and theca cells (tc) of growing follicles, in macrophages (m) in the interstitium and in the antrum of atretic pre-ovulatory follicle (PF). CL = corpus luteum. (**B**) Pre-ovulatory follicle at pro-oestrus. The BrdU labelling index was determined in the designated areas with the help of an eyepiece with a grid. (**C**) Pre-ovulatory follicle at P+14. (**D**) Ovary at P+72. Many BrdU-positive cells were found in growing follicles, whereas in the pre-ovulatory follicle (PF) only a few cumulus cells were immunostained. Scale bar = 100 µm.

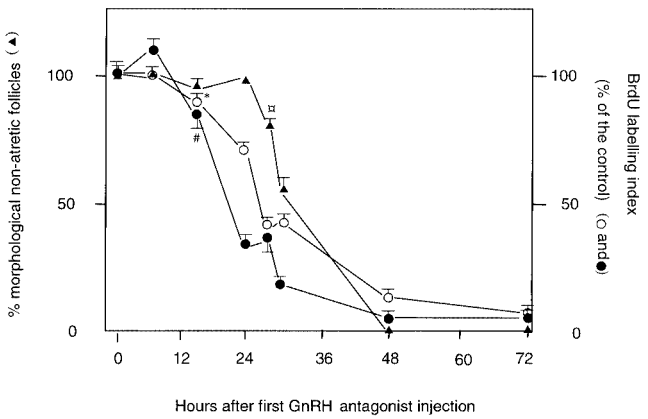

Figure 4. Percentage of morphologically non-atretic pre-ovulatory follicles (▲) and 5-bromo-deoxyuridine (BrdU) labelling index of granulosa cells from pre-ovulatory follicles at different time-points after gonadotrophin-releasing hormone antagonist treatment as a percentage of the BrdU labelling index at 0 h (control). Animals were injected with BrdU 2 h before death and the ovaries were processed for detection of BrdU incorporation. The BrdU labelling index decreased during the process of atresia. This decrease was delayed in granulosa cells in the vicinity of the oocyte (o) compared to the labelling index of other granulosa cells (○). Values are given as mean percentages ± SEM (n = 8). *, #, (●): value of indicated time-point and time-points following this time-point differ significantly ($P < 0.05$) from the value of time-point 0.

Table II. 5-Bromo-deoxyuridine labelling index of cumulus or non-cumulus granulosa cells of rat pre-ovulatory follicles

Time after GnRH antagonist treatment (h)	Animal no. 1[a]		Animal no. 2	
	Cumulus[b]	Non-cumulus[b]	Cumulus[b]	Non-cumulus[b]
0	22 ± 3	30 ± 2	24 ± 2	30 ± 1
7	26 ± 3	30 ± 2	24 ± 2	29 ± 2
14	20 ± 3	26 ± 2	19 ± 2	26 ± 1
24	8 ± 2	21 ± 2	7 ± 1	20 ± 1
27	8 ± 1	12 ± 1	9 ± 1	12 ± 1
31	4 ± 2	13 ± 2	4 ± 1	12 ± 1
48	0 ± 1	4 ± 1	1 ± 1	3 ± 1
72	1 ± 1	1 ± 1	1 ± 1	1 ± 1

[a]In each animal four pre-ovulatory follicles were investigated.
[b]Mean ± SEM.
GnRH = gonadotrophin-releasing hormone.

of isolated follicles in the presence of GnRH antagonist did not affect steroid hormone production (unpublished observations). Furthermore, in hypophysectomized rats treated with recombinant FSH, an effect of GnRH antagonist on the total number of antral follicles and on the percentage of atresia was not observed (Uilenbroek *et al*., 1996). In addition, the morphological changes found after GnRH antagonist treatment in the present study were not different from those reported after pentobarbital treatment (Braw and Tsafriri, 1980;

Uilenbroek *et al*., 1980) or after hypophysectomy (Talbert *et al*., 1951; Braw *et al*., 1981).

It has been shown before, both by autoradiographic methods and in-situ detection methods, that internucleosomal DNA fragmentation occurs in granulosa cells of follicles undergoing atresia (Hughes and Gorospe, 1991; Palumbo and Yeh, 1994). Internucleosomal DNA fragmentation was never found in granulosa cells of follicles that are non-atretic as judged by morphological criteria. The in-situ TUNEL-assay was used to time the occurrence of internucleosomal DNA fragmentation during the process of atresia in pre-ovulatory follicles. The first sign of internucleosomal DNA fragmentation in granulosa cells was detected just 3 h (P+24) prior to the morphological changes that occur in granulosa cells during the process of atresia. In a comparable study, in which rats were hypophysectomized on the morning of pro-estrus, it was found that DNA fragmentation (detected by autoradiography) and the presence of apoptotic granulosa cells (nuclear condensation detected by DAPI staining) occurred concomitantly by 8 h after hypophysectomy (Nahum *et al*., 1996). Additional studies show that TUNEL-positive granulosa cells are only found in atretic follicles (Li *et al*., 1998; Kim *et al*., 1998). Since the time between the occurrence of internucleosomal DNA fragmentation and the appearance of the first morphological signs of atresia is so short, it is concluded that internucleosomal DNA fragmentation detected by TUNEL assay is not suitable as a marker of future atresia. In addition, the sensitivity of the TUNEL assay is a point of consideration. In the experiments in the current study, background staining showed variation, for which no exact cause could be identified. A possible explanation could be that the variable amount of background staining was the result of some general endonuclease activity during the execution of the TUNEL assay. Although the signal measured by the TUNEL assay was very clear in follicles at later time-points, the variable background signal interfered with the faint signal in follicles found at P+24. Since DNA fragmentation not only occurs during apoptosis but also, for example, during necrosis and DNA repair, it has to be noted that the present TUNEL staining method is not specific for apoptosis. However, since apoptotic nuclei contain a far greater degree of DNA fragmentation than nuclei in any other situation, a stronger TUNEL staining will be found in apoptotic cells than in non-apoptotic cells (Ansari *et al*., 1993).

As an alternative to apoptotic markers, BrdU labelling was used as a measure of proliferative activity of granulosa cells in the pre-ovulatory follicles that undergo atresia. It appeared that the BrdU labelling index was significantly decreased at P+14 and at the time-points thereafter. In granulosa cells in the vicinity of the oocyte a similar but delayed pattern was observed, indicating that these granulosa cells proliferate longer than granulosa cells more distant from the oocyte. In addition, the granulosa cells in the vicinity of the oocyte are the last to go into apoptosis. Thus, it appears that a clear difference exists between the granulosa cells that are close or distant from the oocyte and that the BrdU labelling index and atresia are well correlated. In pig follicles, for example, the cumulus cells never go into apoptosis during atresia in antral follicles (Manabe *et al*., 1996). The finding that the BrdU labelling index of granulosa cells decreases during follicular atresia corresponds well to earlier observations (Gaytan *et al*., 1996). The authors of this study estimated the proliferative capacity of different ovarian compartments in cycling rats by BrdU labelling and they found that in atretic non-pre-ovulatory follicles the number of proliferating granulosa cells decreased as compared to the number in healthy follicles of the same size, even in type Ia atretic follicles which exhibit only a few pyknotic granulosa cell nuclei. Similar results were found in early atretic follicles in the ewe (Jablonka-Shariff *et al*., 1996). In the present study, it was found that those follicles destined to become atretic, but do not yet show any morphological sign of atresia, already have a decreased BrdU labelling index of their granulosa cells. Thus, it may be concluded that the first step leading to follicular atresia is a decrease of proliferation. Indeed, in rat granulosa cells the control of granulosa cell apoptosis may involve both cell cycle arrest at G_1/S (Tsang *et al*., 1999), as has been found in other cell types (Scott *et al*., 1986; Ueno *et al*., 1997). The correlation between follicular atresia and decreased proliferation of the granulosa cells was also found in a study by Li *et al*. (1998).

In conclusion, BrdU labelling may be used as an upstream marker of atresia in follicles that do not show morphological signs of atresia. This measure of proliferative activity should be further developed to be used as a functional parameter in the study of regulation of follicular atresia. The present animal model can be used to try to identify additional upstream markers of follicular atresia.

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