

Original article

Subtypes of active cell death in the granulosa of ovarian atretic follicles in the quail (*Coturnix coturnix japonica*)

K D'Herde, B De Prest, F Roels

Department of Human Anatomy, Embryology and Histology, University of Ghent,
Godshuizenlaan 4, B-9000 Ghent, Belgium

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Summary — Follicular atresia in the ovaries of Japanese quail was studied by cytochemistry and electron microscopy. Three different types of cell death coexisted in the granulosa. A large number of cells showed signs of apoptosis. The DNA fragmentation in these cells was demonstrated in a previous study using in situ end-labeling. A second and non-negligible type of cell death consisted of extensive autophagocytosis of the cytoplasm occurring simultaneously with late nuclear alterations. Finally, a few detached cells displayed cytoplasmic disintegration and small irregular clumps of chromatin condensation indicative of primary cell necrosis. Apoptotic versus autophagic cell death revealed a different pattern of acid phosphatase activity (lysosomal versus cytoplasmic). We propose that these observations may be linked to the existence of distinct subpopulations in the granulosa as has been shown by others. This study confirms the biochemical data on granulosa cell death, but demonstrates that apoptosis is not the exclusive mode of active cell death in follicular atresia.

apoptosis / programmed cell death / follicular atresia / lysosome / quail

Résumé — **Subtypes de mort cellulaire active dans les follicules atrétiques de la caille japonaise (*Coturnix coturnix japonica*)**. L'atrésie folliculaire dans l'ovaire de la caille japonaise a été étudiée par microscopie électronique et cytochimie. Trois types de mort cellulaire coexistent dans la granulosa. Un grand nombre de cellules montrent les signes d'apoptose, dont nous avons démontré dans une étude précédente la fragmentation de l'ADN par la technique appelée in situ end-labeling. À côté de l'apoptose certaines cellules présentent une autophagie marquée du cytoplasme accompagnée de changements nucléaires tardifs. Finalement un petit nombre de cellules montre la désintégration cytoplasmique généralisée et la condensation de chromatine en petites masses irrégulières, typique de la nécrose primaire. En accord avec cette diversité morphologique une localisation hétérogène de l'activité des phosphatases acides (lysosomiale et cytoplasmique) a été décelée. Ces différentes formes de mort cellulaire peuvent être liées à l'existence de différentes populations de cellules dans la granulosa, comme cela a été démontré par d'autres. Cette étude confirme les données biochimiques, mais souligne le fait que l'apoptose n'est pas l'unique mécanisme de mort cellulaire active dans l'atrésie folliculaire.

apoptose / mort cellulaire programmée / atrésie folliculaire / lysosomes / caille

INTRODUCTION

Apoptosis is induced in terminally-differentiated cells of hormone-dependent tissues such as the mammary gland and the prostate in the absence of the appropriate trophic hormones. This results in the regression of the tissue (Bursch et al, 1990a). Similarly, ovarian atrophy (atresia) can be induced in vertebrates by various techniques affecting the gonadotrophin levels (Saidapur, 1978; Gilbert et al, 1981; Tanabe et al, 1981; Yoshimura and Tamura, 1985). Food deprivation or starvation has often been used for this purpose (Verheyen et al, 1987; Saidapur et Prasadmurthy, 1988). According to Greenwald and Terranova (1988), atresia in the mammalian ovary seems to be initiated by the death of the granulosa cells (GCs). Other authors, however, claim that the appearance of pyknotic nuclei in the granulosa layer of atretic avian follicles is neither an early or an obligatory event (Gupta et al, 1988).

Recent advances suggest that GC death in mammals represents an example of apoptosis. Besides there being some morphological similarities (Hay et al, 1976; Byskov, 1978; O'Shea et al, 1978; Tsafirri and Braw, 1984; Hirshfield, 1989), biochemically, the distinctive ladder pattern of DNA fragments has been found during electrophoresis of GC lysates, a result of the characteristic cleavage of genomic DNA into oligonucleosomal-length fragments of 180–200 base pairs, or multiples thereof, by endogenous nuclease (Hughes et Gorospe, 1991; Tilly et al, 1992; Tilly et al, 1995).

The biochemical identification of apoptosis was performed on GC lysates of immature rats treated with PMSG (pregnant mare's serum gonadotrophin) from which, subsequently, gonadotrophic support was withheld. Moreover, GC lysates of untreated animals also showed internucleosomal DNA cleavage, suggesting that cell death also occurs by apoptosis under spontaneous

conditions. Several recent reports on follicular atresia in mammals concern the in situ labeling of DNA fragments in the GCs of induced or spontaneous atretic follicles (Gavrieli et al, 1992; Billig et al, 1993; Billig et al, 1994). In the avian ovary biochemical and morphological studies of atresia are contradictory, however. The use of autoradiographic analysis has revealed extensive apoptotic DNA cleavage in both the GCs and theca cells of atretic chicken follicles. This was absent in the GCs and theca cells of healthy follicles (Tilly et al, 1991). In a previous study we confirmed these biochemical data at the single cell level by means of two in situ end-labeling techniques (D'Herde et al, 1994).

In contrast, the only ultrastructural study published thus far concerning cell death in avian atretic granulosa (Kovacs et al, 1992) suggests that the dominant type of cell death in the granulosa of atretic goose follicles is 'cell necrosis' (as defined by Clarke, 1990). Cell necrosis is characterized biochemically by the cleavage of DNA into fragments displaying a continuous spectrum of sizes, or presenting no fragmentation at all (Schwartzman and Cidlowksi, 1993). This discrepancy between morphological and biochemical data may suggest that more than one cell death pattern is involved.

In order to elucidate the mode of GC death during atresia, a study was undertaken in which induced atresia was studied ultrastructurally in quail ovaries. In order to evaluate the role of lysosomes during cell death in this model, acid phosphatase cytochemistry was utilized.

MATERIAL AND METHODS

Experimental procedure

Twenty five daily-laying Japanese quail (*Coturnix coturnix japonica*) reared under continuous light

were housed in individual cages. Water was available ad libitum but they were deprived of food for 72 h. This regimen is used in poultry husbandry for the induction of forced molting, which is then followed by an increase in egg production after the resumption of feeding, due to the rejuvenation of the hen (Tanabe et al, 1981). Controls were given food (fresh lettuce and complete breeding food (HENS)) and water ad libitum during the same experimental period. We examined the preovulatory follicles in rapid growth phase (the follicle hierarchy: F_1 , F_2 , F_3 , F_4 and F_5 according to the nomenclature of Gilbert, 1971). Under natural conditions the ovaries of daily-laying birds have a large population of atretic follicles in the size class of small yolky follicles (Gilbert et al, 1985); for the quail these follicles have a diameter of 1–6 mm. Obviously these small atretic follicles are in various stages of atresia. In contrast, spontaneous atresia in the larger follicles constituting the follicle hierarchy (6–19 mm) is a rare event. By inducing atresia in the population of large follicles, which under normal conditions are destined to ovulate, the process is synchronized in these follicles. The fact that after shorter starvation periods of 60 h no atresia was detected in the follicle hierarchy, while it has been shown to take approximately 24 h to develop macroscopic signs of bursting atresia (Gilbert, 1979) indicates that we studied follicles involved maximally since 24 hours in an atretic process. This approach made it possible to study the fine structure of a large number of atretic follicles in an early stage of degeneration. The atretic follicles were identified macroscopically by the occurrence of hemorrhagic areas on either side of the stigma and by their irregular convoluted surface (Gilbert et al, 1985).

Light microscopy

Whole follicles were fixed in Bouin's solution or 4% buffered formaldehyde. Paraffin sections were stained either with hematoxylin and eosin or with the Feulgen nuclear reaction for DNA.

Apoptotic cells were identified by their characteristic morphological features as seen on 3 μ m H & E stained sections. Apoptosis was scored in ten randomly-selected microscopic fields at 100x magnification and expressed as the number of apoptotic cells per number of total nuclei counted. A total of 400–1 500 granulosa nuclei were counted per atretic follicle. Small groups of apoptotic bodies were counted as remnants of one

apoptotic cell. As has been shown previously, this method is more sensitive than counting on in situ end-labeled sections (D'Herde et al, 1994). Autophagic cell death was scored in β -glycerophosphatase-stained 2- μ m sections and expressed as the number of cells with diffuse cytoplasmic phosphatase activity versus the total number of cells.

Transmission electron microscopy

After decapitation of the animals, the follicles were rapidly dissected from the ovaries and fixed by immersion in a compound aldehyde fixative as described previously (D'Herde and Vakaet, 1992). After an initial fixation in toto for a three-day period, fragments of follicle wall with adherent yolk were dissected then underwent further fixation for 2 days. After fixation the tissue was rinsed in 0.1 M cacodylate buffer containing 7.5% sucrose. Postfixation was carried out with 2% OsO₄ buffered in 0.1 M cacodylate. Block staining with uranyl acetate (0.5% in veronal acetate, pH 5) was carried out for 30 min at room temperature before embedding. The tissue was embedded in LX (Ladd, Burlington, Vermont, USA). Semi-thin (2 μ m) sections were contrasted with toluidine blue. Ultra-thin sections were stained with uranyl acetate and lead citrate; uncontrasted sections were also examined.

Enzyme cytochemistry

A fixative consisting of 2% glutaraldehyde buffered with 0.1 M cacodylate and 0.1% CaCl₂ was used. After rinsing, 7- μ m cryostat sections (Dittes-Duspiva cryostat) were placed on APS (aminopropylsilane)-coated slides. These sections were incubated for light microscopy according to Lojda et al, 1979. The substrate was naphthol-AS-TR phosphate (Sigma) and the coupler was hexazotized *p*-rosaniline. After overnight postfixation in 10% formaldehyde containing 1% CaCl₂, the sections were mounted in water-soluble medium (Gurr). This stain is quantitative (Roels et al, 1984).

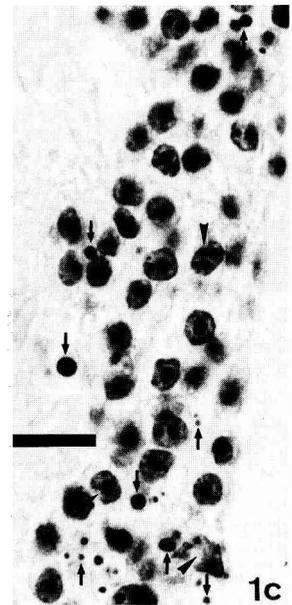
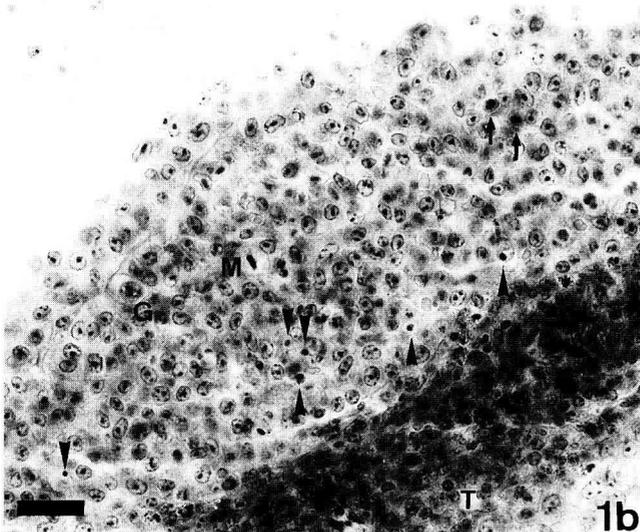
For electron microscopy, 60- μ m chopper sections (tissue sectioner Smith and Farquhar) were rinsed overnight in buffered cacodylate containing 7.5% sucrose. The acid β -glycerophosphatase activity was then revealed according to the Gomori-lead procedure modified by De Jong et

al (1979). The reaction product was visualized for light microscopy by treatment with ammonium sulfide. Two controls were included: incubations were performed in the presence of the inhibitor

NaF (0.01 M) and in the absence of substrate. After rinsing the chopper sections post-osmication, en-bloc staining and embedding of the tissue was carried out as described above.



Fig 1. (a) Characteristics of spontaneously bursting atresia in a small yolk follicle: presence of yolk in the lacunar spaces (L) of the tunica superficialis, multilayered aspect of the granulosa (G). In the degenerate oocyte (O) detached granulosa cells with pyknotic nuclei are recognized; T: theca; H & E staining; bar: 100 μ m. (b) Detail of the multilayered granulosa of fig 1a. Note the presence of several apoptotic bodies (arrowheads), nuclei with condensed chromatin (arrows) and one metaphase (M); G: granulosa; T: theca; H & E staining; bar: 20 μ m. (c) Detail of the granulosa of a starvation-induced atretic F4 follicle, Feulgen staining. Note the numerous Feulgen positive apoptotic bodies (arrows), some of them lying in clusters; nuclei with irregular contour (arrowheads); bar: 10 μ m.



RESULTS

Light microscopy

The histology of the induced atresia in the quail follicles constituting the follicle hierar-

chy was comparable to naturally-occurring atresia in the smaller vitellogenic follicles as described by other authors (Gilbert, 1979; Gupta et al, 1988). Atresia of these follicles is invariably associated with rupture of the follicular wall and extrusion of yolk through the rupture site into the lacunar spaces of

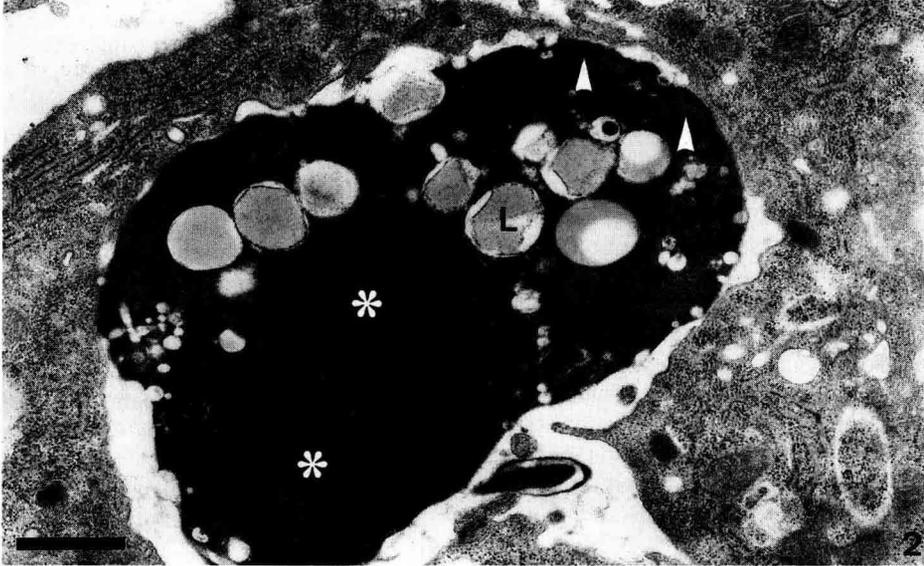
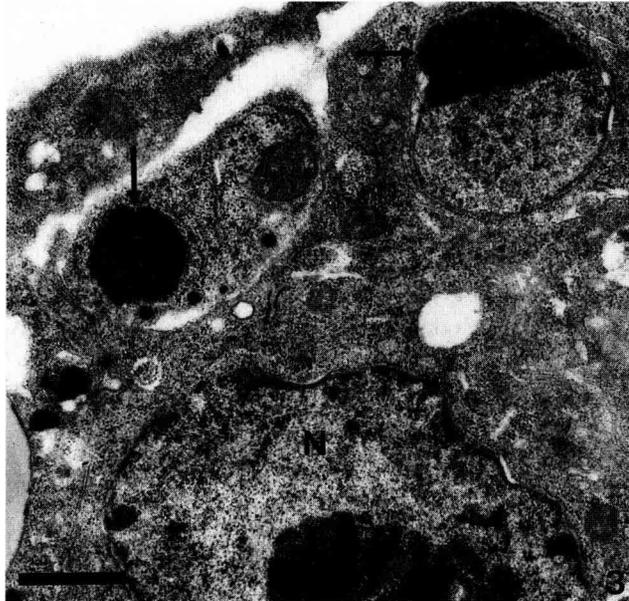


Fig 2. Granulosa of atretic follicle (F_4), apoptotic cell separated from its neighbours and showing cytoplasmic condensation and margination of chromatin (asterisks). Note two ER cisternae contiguous with the plasma membrane (white arrowheads); L: lipid droplet; bar: 1 μ m.

Fig 3. Granulosa of atretic follicle (F_4). Two apoptotic bodies can be identified by the condensed texture and crescent shape of their chromatin (arrows); their cytoplasmic structure is not different from the adjacent cell containing a normal nucleus (N); bar: 1 μ m.



the tunica superficialis (Callebaut, 1979). In the oocyte, cellular debris and disorganization of yolk are seen (fig 1a).

In contrast to the one-layered granulosa of healthy follicles, the granulosa layer of induced atretic follicles had a multilayered aspect or was folded. In some places, sheets of GCs were detached from the basement membrane. In the hematoxylin–eosin-stained paraffin sections, the occurrence of extensive cell loss was revealed by the presence of numerous apoptotic bodies. These contained rounded and structureless chromatin masses of divergent sizes but always smaller than a normal nucleus. These bodies often occurred in clusters and were Feulgen-positive (fig 1b, c). Apoptotic bodies without a nuclear component were inconspicuous at the light microscopical level. Several granulosa nuclei had an irregular outline. In the degenerated oocyte, cells with eosinophilic cytoplasm and clearly pyknotic nuclei were found, in addition to cell ghosts with karyolytic nuclei.

Electron microscopy

The classical features of apoptosis (Wyllie, 1981; Bowen and Bowen, 1990) were obvious. We found isolated GCs with very dark cytoplasm containing masses of free ribosomes and clumped chromatin accumulating at the nuclear rim. Images suggesting fusion of dilated endoplasmic reticulum cisternae with the cell surface, a morphological feature of apoptosis (Wyllie, 1993), were encountered (fig 2). More often, small cell fragments with typical apoptotic nuclear remnants (ie, margination of chromatin in large crescent-shaped caps or condensed chromatin occupying the entire area within the nuclear envelope) displayed remarkably normal cytoplasmic structure without packing the cellular organelles (fig 3). Some apoptotic bodies were found inside the degen-

erated oocyte, but most were present in the heterophagic vacuoles of intact GCs. In these vacuoles, progressive stages of degradation of the apoptotic bodies could be recognized (fig 4). Cellular and nuclear fragmentation was illustrated by the presence of large cell protuberances containing a nuclear fragment.

Some GCs were nearly filled with several smaller autophagic vacuoles (fig 5a–c) that were distinguishable from the above-mentioned heterophagic vacuoles by their contents and diameter. These vacuoles contained mitochondria, ribosomes or fragments of endoplasmic reticulum of the same electron density as the surrounding cytoplasm and had a mean diameter of 0.5–1 μm . Unlike the heterophagic vacuoles, which were bound by a single membrane, some autophagic vacuoles were delimited by a pair of membranes as has been previously reported (Holtzmann, 1989). Occasionally fusion between an autophagic vacuole and a dense body was observed. Typically the membrane of the vacuole became more concave at the contact site (fig 5c), as has been observed by others (Baba et al, 1994). The GCs with autophagic vacuoles had an enlarged Golgi apparatus, their nuclei had typically irregular contours, and in the advanced stages, chromatin condensation was manifest. The condensed chromatin was centrally located in a large mass or in addition scattered in small aggregates at the nuclear periphery. This chromatin condensation was distinct from what was seen in the apoptotic nuclei, where the chromatin segregate in clearly-defined masses that lie against the nuclear membrane. At the level of the plasma membrane, both apoptotic and autophagic cells showed blebbing, disappearance of intercellular junctions and loss of microvilli. Fusion of several autophagic vacuoles generated large areas of degraded cytoplasm. The autophagic process was thus more extensive than that associated with cytoplasmic turnover in

healthy cells (for a review of the latter, see Glaumann et al, 1981). Groups of GCs were present in the degenerate oocyte. Their chromatin had moved to the margin in multiple small aggregates or had disappeared. The disintegration of their cytoplasmic organelles was striking. They contained swollen mitochondria whose internal cristae had disappeared. Their endoplasmic reticulum and nuclear envelope were dilated and most notably their ribosomes had disappeared. Neutrophils were typically located near to these dead cells. In contrast to the apoptic and autophagic modes of cell death, this third type of degeneration was never found in the epithelium itself. This pattern did not represent a phenomenon of secondary necrosis of the apoptotic bodies (Wyllie et al, 1980), as no intermediate stages were observed.

In all the epithelial cells, a remarkable accumulation of lipid droplets was observed. This lipid accumulation did not occur in conjunction with any ultrastructural features of steroidogenesis, ie, mitochondria with tubular cristae or agranular endoplasmic reticulum (Kimura et al, 1989).

Enzyme cytochemistry

The granulosa of healthy yellow follicles displayed moderate activity in their primary and secondary lysosomes as revealed by the naphtol-AS-TR acid phosphatase technique and by the modified Gomori technique. The distribution of lysosomes was homogeneous throughout the epithelium. An increase in both enzyme activity levels was observed in atretic granulosa. This increased activity

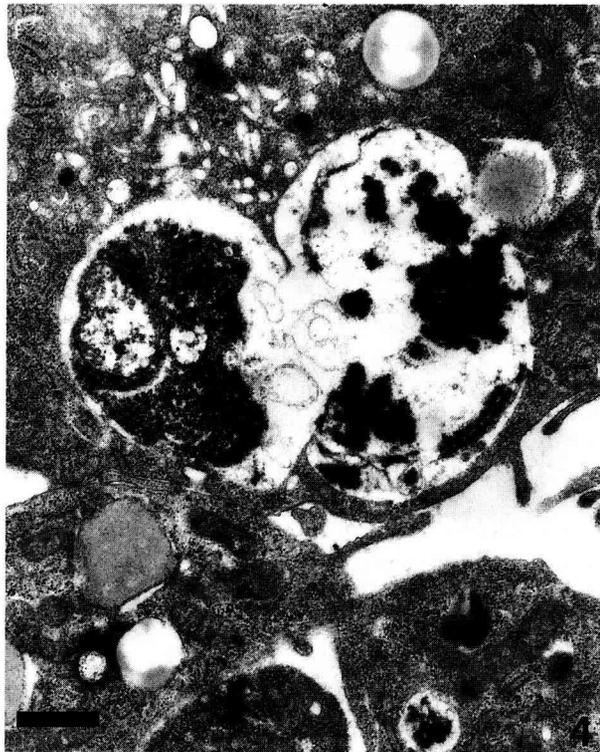


Fig 4. Heterophagic vacuole delimited by a single membrane containing a partly-digested apoptotic body constituted by condensed cytoplasmic and nuclear elements (compare to fig 5); bar: 500 nm.

was not only due to particulate (lysosomal and vacuolar) activity, but also to a strong cytoplasmic reaction in many cells. A mosaic pattern of GCs with moderate or very high β -glycerophosphatase staining was seen under light microscopy (fig 7).

At the ultrastructural level, product of the acid β -glycerophosphatase reaction was found in the heterophagic vacuoles containing apoptotic bodies, the autophagic vacuoles, the Golgi cisternae and primary

lysosomes, and the cytoplasm of non-necrotic cells. The latter displayed the nuclear morphology previously described for autophagic cell death; this reaction product was diffuse, ie, not particle-bound.

Although surrounded by cytoplasmic acid phosphatase activity, the mitochondria retained a normal morphology (fig 8). Some autophagic vacuoles showed an annular pattern of phosphatase activity. Intermingled with the degenerating granulosa cells were

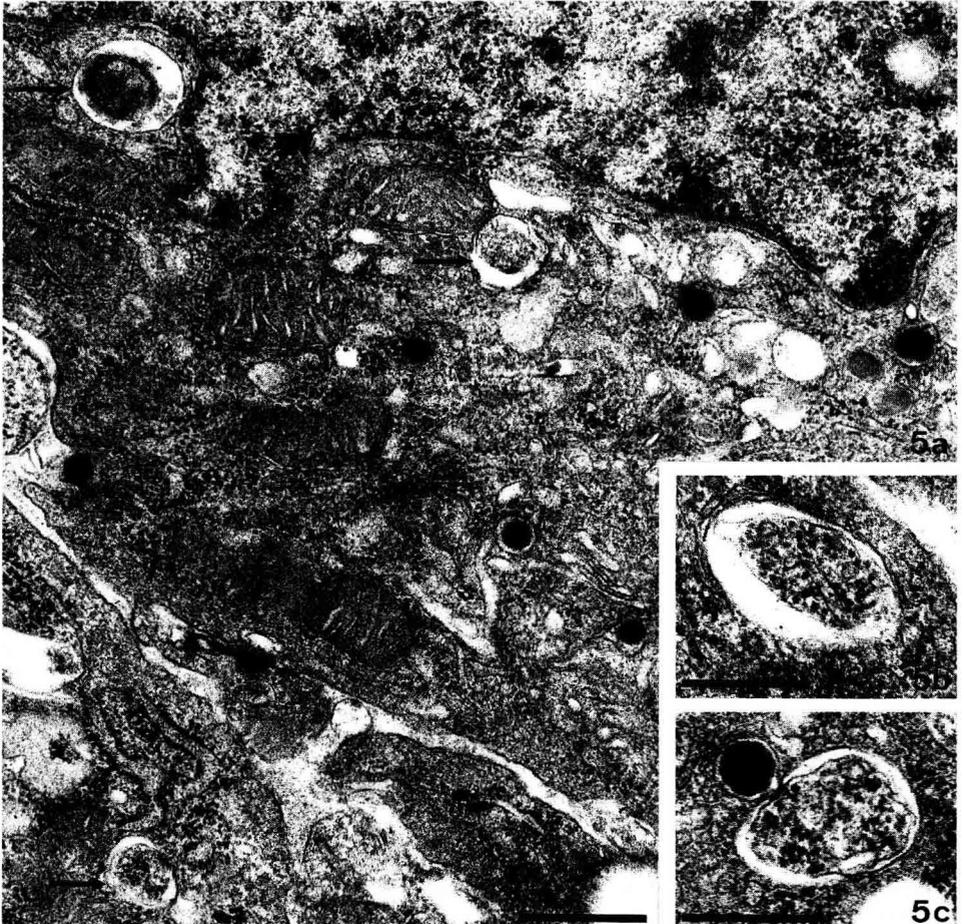


Fig 5. (a) Several autophagic vacuoles (arrows) in two adjacent granulosa cells. Note the irregular contour of the nucleus and the presence of several dense bodies with membrane and halo (most probably lysosomes). (b) Autophagic vacuole delimited by a pair of membranes. (c) Autophagic vacuole fusing with a dense body (most probably a lysosome); bars: 500 nm.

some large round cells that displayed numerous acid phosphatase positive granules.

Frequencies

Counts in five follicles showed an apoptosis frequency of $7.4 \pm 2.4\%$ SEM. Autophagic cell death represented 25, 20, 3 and 2% of the granulosa cells. High frequencies of autophagy did not coexist with high frequencies of apoptosis, suggesting that the two processes were usually asynchronous. Figure 8 shows that both pathways can occur simultaneously in a single follicle.

These counts do not correctly reflect the relative contributions of these two different cell death pathways in this system, however, as one has to take into account the difference in duration of the visible stage of each process. For apoptosis it is known to be approximately 3 h (Bursch, 1990b), making it possible to calculate the tissue regression per day based on the percentage of apoptotic nuclei at a given time. For our model (apoptotic nuclei: 7.4%) these calculations yielded a regression of ca 45% per day.

Primary cell necrosis was the fate of a minority of the granulosa cells.

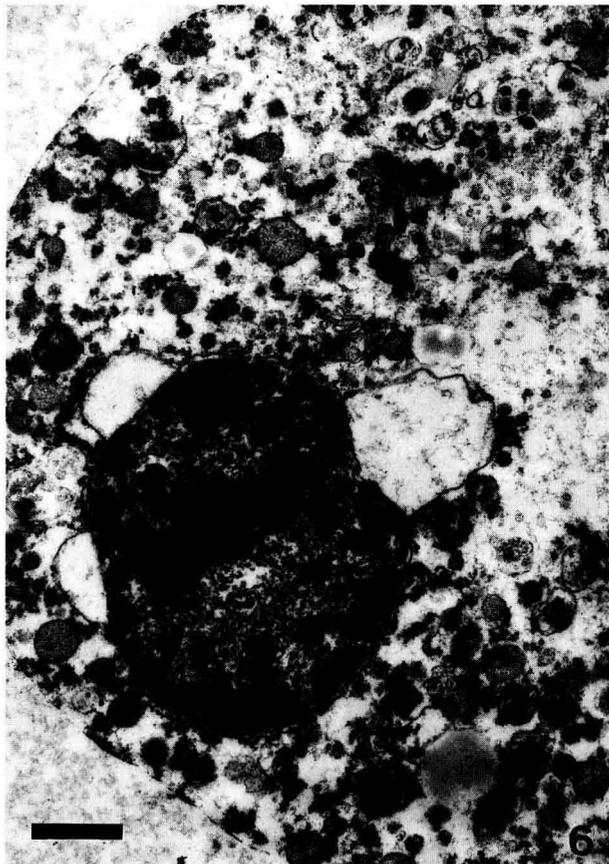
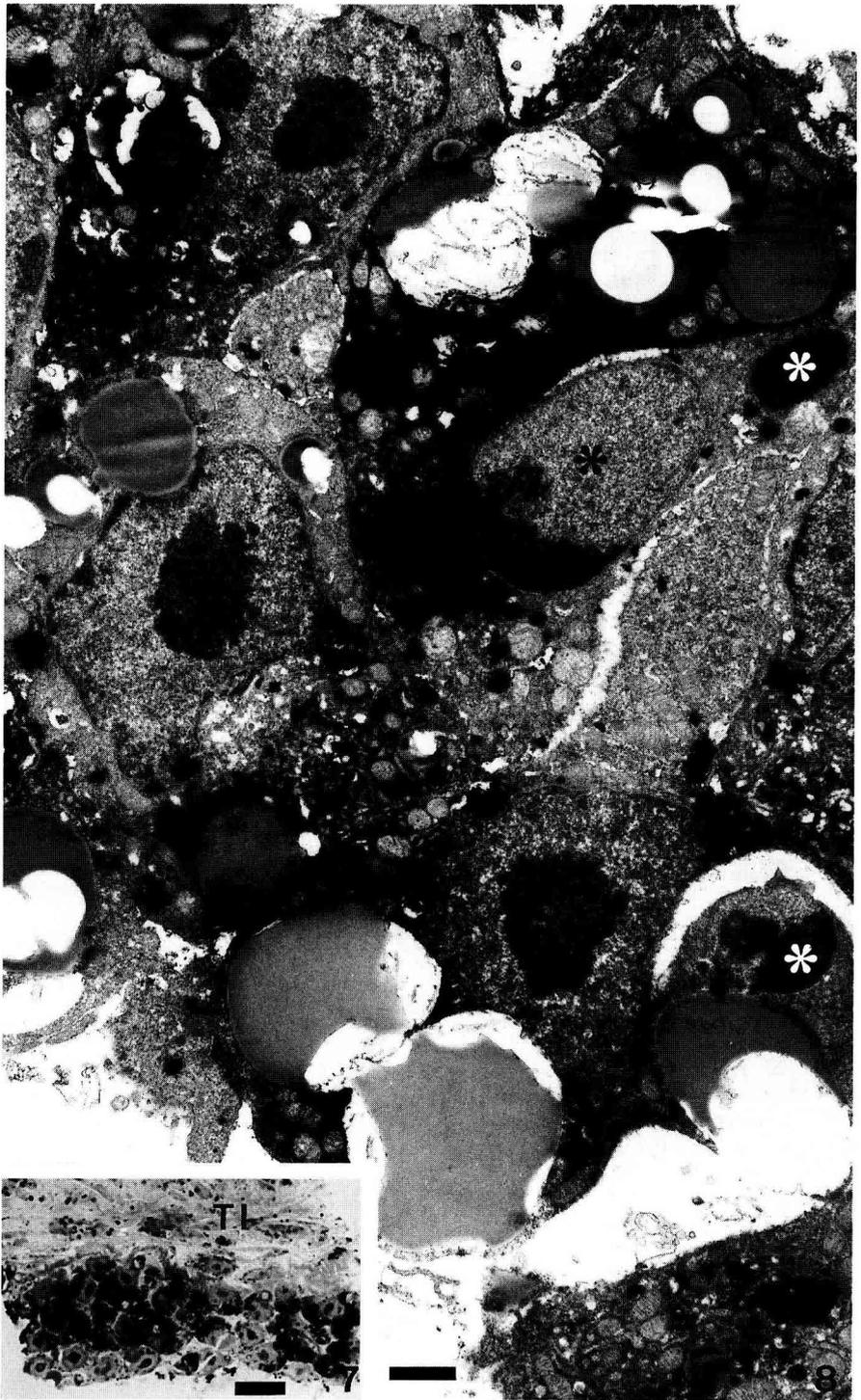


Fig 6. Appearance of a necrotic granulosa cell detached from the epithelium and lying in the degenerate oocyte (compare to fig 1a); nucleus showing margination of chromatin in small dense aggregates and dilatation of the perinuclear cisterna; note the disintegration of the surrounding cytoplasm; bar: 1 μ m.



DISCUSSION

Our observations of the ultrastructure of induced cell death in the granulosa only partly confirm the identity of apoptosis and programmed cell death as has been previously suggested in many publications.

Kerr et al (1972) and Wyllie (1981) propose a radical dichotomy between 'necrotic cell death', which is supposed to occur only in pathological situations, and apoptosis, which occurs in several natural and some pathological situations. In a landmark paper, Schweichel and Merker (1973) described three different types of active cell death in embryonic tissues: apoptosis, autophagic cell death and, finally, nonlysosomal disintegration. Recent reports also distinguish several morphological types of programmed or active cell death and emphasize that the terms 'programmed cell death' and 'apoptosis' are not interchangeable (Lockshin and Zakeri, 1991; Bowen, 1993; Schwartz et al, 1993; Bursch and Grasl-Kraupp, 1994; Majno and Joris, 1995). Recently a monoclonal antibody BV 2 was isolated which recognizes an antigen expressed during avian programmed cell death in both apoptotic and certain necrotic cells, indicating that these cell-death patterns may share similar biochemical features (Fernandez et al, 1994).

The simultaneous presence of apoptosis and cell necrosis in a tissue was demonstrated in the colonic intestinal epithelium of mice with graft-versus-host disease (Buja et al, 1993). The simultaneous presence of apoptosis and cell death by autophagocytosis was shown in the mesenchymal cells

of the developing heart (Krstic and Pexieder, 1973). We observed three modes of cell death in our *in vivo* model of induced atresia. A large fraction of cells died by apoptosis, but in the same tissue fragment GCs degenerated by autophagocytosis, and a third group of cells, which was a minority, was eliminated by a process similar to descriptions of cell necrosis. The difference between apoptotic cells and autophagic cells was confirmed by the presence of DNA fragmentation in the former (as demonstrated by *in situ* end-labeling), and its absence in the latter: a positive label in the granulosa epithelium being confined to cells displaying apoptotic morphology (D'Herde et al, 1994). That the autophagic process observed in the present study was part of a cell-death pattern and not just a phenomenon of cell atrophy can be inferred from the fact that: 1) the atretic process culminates in the total disappearance of the granulosa epithelium (Forgo et al, 1988); 2) autophagy has not been described as a prelude to apoptosis; and 3) in physiological tissue-remodeling by autophagocytosis, no nuclear changes are described. Alterations at the level of the plasma membrane, which would be related to the loss of hormonal receptors in follicular atresia (Peluso et al, 1980), were not present in physiological autophagocytosis. A dual GC-death pattern was reported by Peluso et al (1981) in an *in vivo* model where atresia was induced in rat by either antiestrogen or antiandrogen. Apoptotic and necrotic GCs were found to coexist in the atretic granulosa.

Similar results on GC death were observed in an *in vitro* model using serum-

Fig 7. Abundant acid β -glycerophosphatase activity in part of the granulosa cells. Compare to the theca interna (TI) showing only small reactive granules; bar: 10 μ m.

Fig 8. Heterogeneous pattern of acid β -glycerophosphatase staining in atretic granulosa. Note three nuclei with apoptotic configuration (asterisks); the other nuclei show irregular contours and chromatin condensation; three granulosa cells, two of which seem to engulf apoptotic bodies, show heavy reaction product in the cytoplasm surrounding mitochondria and fat droplets, as well as in some lysosomes; their nuclei have a large central chromatin mass which is characteristic of GCs displaying autophagocytosis; bar: 1 μ m.

free cultures: apoptosis was shown to occur predominantly in the subpopulation of large steroidogenic GCs (Luciano et al, 1994; Peluso and Pappalardo, 1994). For mammals and birds, the existence of two distinct subpopulations of GCs is firmly established (Marrone and Crissman, 1988; Sanbuissho et al, 1993). Kovacs et al (1992), who studied the ultrastructure of spontaneous atretic ovarian follicles of the goose (*Anser anser f domestica*), concluded that the overwhelming majority of goose atretic follicles exhibit the characteristics of necrotic-type disintegration; we cite: 'accumulation of lipid droplets and dense irregular masses of unknown origin, dilation and disintegration of RER cisterns, swelling of mitochondria, nuclear indentations and detachment of the cells from the basal lamina. In some atretic follicles a small number of cells, characterized by extremely dense cytoplasm, were described as apoptotic. However nuclear fragmentation and uptake of apoptotic bodies in heterophagosomes were not observed. As demonstrated by Bursch et al (1990b), phagocytosis and digestion of apoptotic bodies occur rapidly and it is estimated that they remain visible under light microscopy for only a few hours, so that the process can be remarkably inconspicuous. The different ratio of apoptosis versus cell necrosis found by Kovacs et al (1992) can probably be explained by the different experimental conditions. As they were studying spontaneous atresia, they analyzed, on average, older phases of the process than we did in induced atresia.

We speculate that cell necrosis, which we observed in the innermost GCs that were furthest from the blood vessels, was not programmed in the genetic sense but was secondary to the ischemia resulting from thecal hemorrhages. Indeed, it has been demonstrated that disruption of the thecal blood vessels and leakage of blood to the pericapillary spaces precedes changes that are visible under the light

microscopic in the granulosa epithelium during spontaneous follicle atresia (Gilbert et al, 1985).

The role of acid hydrolases in programmed cell death is both complicated and controversial and has been dealt with in several reviews (Bowen and Bowen, 1990; Clarke, 1990). There is general agreement about the involvement of acid hydrolases following cell fragmentation in the processes of phagocytosis and digestion by neighboring cells. There is little doubt that the presence of these phagosomes elicits new synthesis of hydrolases; an increase in hydrolase activity in response to the ingestion of apoptotic bodies has been reported in neoplastic epithelial cells (Bowen and Bowen, 1990). We observed an increase in atretic granulosa with both substrates. New synthesis of hydrolases could explain our finding of β -glycerophosphatase activity in Golgi cisternae, which was absent in the granulosa of healthy follicles.

Elevated autophagic lysosomal activity has been widely reported in a range of cells and tissues, often leading to an apparently programmed cell death but not following the profile of classical apoptosis (Beaulaton and Lockshin, 1982; Clarke, 1990; Lockshin and Zakeri, 1994). Autophagic vacuoles are sometimes reported to break down and spill their contents into the cytoplasm. Cytoplasmic acid phosphatase activity is classically reported in connection with programmed cell death by cell autolysis in invertebrates (Bowen and Ryder, 1974). In vertebrate models, cytoplasmic acid phosphatase has been reported in thymocyte and tumour cell apoptosis (Bowen and Bowen, 1990). In the case of cell necrosis it is known that the destruction of membrane integrity leads to the release of lysosomal enzymes (Kerr and Harmon, 1991). In our model, however, the appearance of cytoplasmic activity was quite dramatic in non-necrotic and non-apoptotic GCs, most prob-

ably confined to the cells undergoing autophagocytosis (fig 8). The presence of cytoplasmic acid phosphatase reaction product has been interpreted by Hoefsmit et al (1985) as an artifact produced during the post-incubation wash procedure, owing to the continued liberation of phosphate ions from residual phosphatase activity and endogenous substrate in the lysosomes, and to the subsequent diffusion of the phosphate ions to sites where the matrix composition favors precipitation and nucleation of the lead salt. However, with our technique no trace of cytoplasmic acid phosphatase activity was ever observed in healthy follicles or in the theca interna of atretic follicles (D'Herde, 1992).

The large, round, acid phosphatase positive cells found intermingled with GCs were identified as macrophages in accordance with similar observations in the rabbit ovary (Kasuya, 1995).

In conclusion, this study presented evidence for the existence of at least two different pathways leading to active GC death in avian follicular atresia: classical apoptosis and cell death by autophagocytosis. Both the morphological observations and the heterogeneous pattern of acid phosphatase activity support this conclusion. Future studies dealing with the regulation of active cell death in follicular atresia should take into consideration the subtypes, especially since it is anticipated that the induction of gene expression differs in these diverse types of cell death (Bursch and Grasl-Kraupp, 1994).

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