CELL KINETIC STUDIES OF FOLLICULAR ATRESIA IN THE MOUSE OVARY

ANNE GRETE S. BYSKOV

*The Finsen Laboratory, The Finsen Institute,
Copenhagen, Denmark*

(Received 16th April 1973)

Summary. Three stages of follicular atresia have been defined in the juvenile mouse ovary. Using autoradiography, the development and duration of the atretic process has been determined. The three stages of atresia represent consecutive stages in a single atretic process. A large follicle will incorporate [^3H]thymidine into its granulosa cells: the labelling index of such a follicle is about 40%. As atresia sets in and progresses, the labelling index of the granulosa cells changes. This change was followed in autoradiographs prepared at different times after pulse labelling. Follicles in the early stages of atresia a day after label injection showed a higher labelling index than healthy follicles. This apparently paradoxical behaviour seems to result from a re-utilization of DNA freed by fragmented, pyknotic granulosa cells released into the follicular cavity. Labelling curves of follicles at different stages of atresia in relation to time were prepared. A comparison of such curves suggests that the time it takes a large follicle to become atretic is about 4 days.

INTRODUCTION

In the ovary, many follicles start growth but only a few reach the preovulatory stage. Most of them become atretic and disappear.

Follicular atresia in the ovaries of various mammals has previously been described by Deane (1952), Alfert (1955), Lobel, Rosenbaum & Deane (1961), Banon, Brandes & Frost (1964), Guraya & Greenwald (1964), Burkl (1965), and Dalmane (1967). These studies define the atretic follicles morphologically and histochemically but do not elucidate the time sequence of the atresia.

The purpose of the present investigation was (1) to define morphologically the different stages through which a large follicle without signs of atresia passes as it transforms to a completely atretic one, and to investigate whether the stages of atresia are consecutive in the atretic process; (2) to determine the duration of the atretic process using autoradiography after injection of [^3H]-thymidine.

MATERIALS AND METHODS

Animals and preparations

Twenty-four female Bagg mice, aged 28 days, each received a single intraperitoneal injection of 50 μCi [^3H]thymidine. The animals were killed in

groups of four at 1, 24, 48, 72 hr and 6 days after the thymidine injection. An intraperitoneal injection of 2.5% glutaraldehyde buffered with collidine was given immediately after killing the mice by cervical dislocation. After removing the ovaries, the fixation was continued in the same solution followed by post-fixation in 1.33% OsO_4 . The ovaries were embedded in Epon 812 and 600 serially cut sections ($1\text{ }\mu\text{m}$) from each ovary were prepared for autoradiography using Ilford K2 emulsion and exposed for 8 to 18 weeks. After development, the sections were stained for $1\frac{1}{2}$ hr at 25°C with toluidine blue-pyronine.

Autoradiographs were prepared from animals killed 1 hr after injection of [^3H]thymidine (the 'immediate autoradiographs') and 24, 48, 72 hr or 6 days after pulse-labelling (the 'delayed autoradiographs').

The ovaries of 28-day-old mice contain healthy follicles of various sizes as well as follicles in different stages of atresia. To distinguish between follicles without signs of atresia and follicles in different stages of atresia, the following criteria were used: (1) the percentage of pyknotic nuclei among the granulosa cells, (2) the presence of polymorphonuclear leucocytes in the follicles, (3) the presence of cavities in follicles that have less than 200 cells in the largest cross-section, (4) the percentage of labelled cells 1 hr after pulse-labelling with [^3H]thymidine. To evaluate follicles without signs of atresia, all healthy follicles found in the serially sectioned ovary with 200 to 500 cells in the largest cross-section were considered. Atretic follicles that had 25 to 500 cells in the largest cross-section were studied.

Since the number of the large follicles in any one ovary is small (about ten to twelve), the total number of follicles studied in all mice was used to compile the Tables and Text-figures.

Labelling index

The labelling index (LI) of a follicle is represented by the number of labelled granulosa cells expressed as a percentage of all the cells counted in the largest cross-section of the follicle. A nucleus is considered to be labelled when three or more grains are present over its area. The probability that a label of more than three grains is the result of background alone is less than 2%.

The percentage of necrotic cells per total cell number in the largest cross-section was determined. In the atretic follicles, the percentage of labelled necrotic cells per total number of necrotic cells was also determined.

The number of grains per $1600\text{ }\mu\text{m}^2$ over the follicular cavity was counted.

The differences in the LI of follicles were tested by Student's *t* test.

Labelling curves and labelling pattern

All labelling curves were obtained by plotting the mean value of the LI in relation to time (Text-figs 1 and 2). The mean value was derived from the LI of four to six similar follicles. A labelling curve illustrates the LI in morphologically similar follicle stages from ovaries of mice killed at different times after pulse-labelling. The LI of a follicle in a 'delayed autoradiograph' depends therefore on two factors: (1) on the LI at injection time of the follicle from which it is derived, and (2) on the evolution such a follicle underwent during the interval between pulse-labelling and killing of the animal.

TEXT-FIG. 1. Labelling index of non-atretic mouse follicles at different times after a single injection of [^3H]thymidine (i.e. the labelling curve). The vertical lines show the range of the labelling index.

RESULTS

The follicles were divided into four groups: follicles without signs of atresia and follicles in three stages of atresia. The criteria are summarized in Table 1.

Follicles without signs of atresia. In these follicles, all granulosa cells were well

TEXT-FIG. 2. Labelling indices of non-atretic mouse follicles and follicles in different stages of atresia at different times after a single injection of [^3H]thymidine (i.e. the labelling curve). The vertical lines indicate the range of the labelling index of the atretic follicles.

defined and leucocytes were never found (Pl. 1, Fig. 1). A cavity was present in all follicles that had more than 300 cells in the largest cross-section.

Follicles in Stage I atresia. These follicles had up to 20% pyknotic granulosa cells which usually lay close to the follicular cavity (Pl. 1, Fig. 2). The pyknotic nuclei were often fragmented and the pieces were sometimes connected by thin bridges. These fragments sometimes protruded from the cell (Pl. 1, Fig. 3) or lay entirely outside the cell as 'nuclear dots'. Polymorphonuclear leucocytes did not occur, and the basement membrane was intact. A follicular cavity was present regardless of the number of granulosa cells. In 'immediate autoradiographs' the LI was significantly lower ($P < 0.02$) than in non-atretic follicles.

Table 1. Criteria to distinguish between atretic and non-atretic mouse follicles

Type of follicle	% pyknotic granulosa cell nuclei	LI† at 1 hr after pulse-labelling with [^3H]thymidine	Leucocytes present in the follicle	Follicular cavity present
Without signs of atresia	0	25 to 45	—	+/-*
With signs of atresia				
Stage I	5 to 20	7 to 34	—	+
Stage II	5 to 20	0 to 6	+	+
Stage III	5	0	+	—

* Only present in follicles with more than 300 cells in largest cross-section.

† LI, labelling index, for explanation see text.

Follicles in Stage II atresia. Pyknotic nuclei were present as in Stage I, and the 'nuclear dots' were numerous (Pl. 2, Fig. 4). Leucocytes were always seen among the granulosa cells, and the basement membrane was not intact (Pl. 2, Fig. 5). A cavity was present in all follicles and appeared to be larger than in follicles in Stage I atresia. The LI was lower ($P < 0.02$) than in follicles in Stage I atresia in 'immediate autoradiographs'.

Follicles in Stage III atresia. These were shrunken and less than 5% of the granulosa cell nuclei were pyknotic (Pl. 2, Fig. 6). The number of leucocytes and the broken basement membrane were comparable to Stage II atresia. In contrast to Stage I and Stage II atresia, a cavity was never seen. The LI was comparable to that of Stage II atretic follicles in 'immediate autoradiographs'.

The labelling curves

The LI in follicles without signs of atresia at different times after pulse-

EXPLANATION OF PLATE 1

FIG. 1. Two non-atretic mouse follicles (O) and part of a follicle in Stage II atresia (II). Pyknotic nuclei are not seen in the non-atretic follicles, but are numerous (arrows) in follicles in Stage II atresia. $\times 400$.

FIG. 2. Mouse follicle in Stage I atresia (I); several nuclei are pyknotic (arrows) and the follicular cavity is filled with cell debris. Part of a non-atretic follicle (O) and a follicle in Stage II atresia (II) can also be seen. $\times 400$.

FIG. 3. Cavity of a mouse follicle in Stage I atresia with fragments of cells. Parts of a pyknotic nucleus are protruding from a cell (arrows). $\times 4300$.

labelling was usually about 40% (Text-fig. 1) though an occasional follicle might show up to 80% of the cells labelled on the 2nd and 3rd day.

The LI in the atretic follicles varied with time (Text-fig. 2). At 1 hr after injection of the [^3H]thymidine, only the morphologically normal cells but none of the pyknotic nuclei were labelled.

In autoradiographs prepared 24 hr after pulse-labelling, no significant change of LI was noticed, and an equal number of non-pyknotic and pyknotic nuclei in follicles of Stage I atresia were labelled. In Stage II and Stage III, nearly all nuclei remain unlabelled (Text-fig. 2).

Two days after pulse-labelling, all follicles in Stage I atresia showed a marked increase in the LI (48 to 68%) compared to their LI in 'immediate autoradiographs' ($P < 0.002$) and to their LI on the previous day ($P < 0.01$). The LI was also higher ($P < 0.05$) than in non-atretic follicles. Follicles in Stage II and Stage III atresia had 26% and 15% labelled cells, respectively, which were significantly different ($P < 0.02$).

TEXT-FIG. 3. Grain counts as multiple of background over the follicular activity from non-atretic mouse follicles, atretic follicles in Stage I and atretic follicles in Stage II at different times after a single injection of [^3H]thymidine. The vertical lines show the variance (standard deviation) of the counts.

In autoradiographs prepared 3 days after pulse-labelling, all atretic follicles showed a considerably higher LI than on the previous days. The increased LI of Stage I atretic follicles was not significant unlike that of Stage II ($P < 0.02$).

EXPLANATION OF PLATE 2

FIG. 4. Three mouse follicles in Stage II atresia (II) with cavities somewhat larger than in follicles in Stage I atresia. Leucocytes are present (arrows). $\times 400$.

FIG. 5. Leucocyte (L) inside the basement membrane of a mouse follicle in Stage II atresia (see marked area in Fig. 4). The basement membrane is only distinguishable in places (arrow). $\times 4300$.

FIG. 6. Two mouse follicles in Stage III atresia (III), without follicular cavities. The basement membrane (arrows) is hardly recognizable. $\times 400$.

and Stage III ($P < 0.05$) follicles which were significantly different from each other ($P < 0.02$).

Six days after pulse-labelling, the LI in Stage I and II follicles was about 30%, comparable to the LI in follicles without signs of atresia. In Stage III atresia, it had risen to 50%; this differed from the LI of other follicles ($P < 0.02$) and was higher than the LI on the 3rd day after labelling ($P < 0.02$).

The labelling over the follicular cavity

Autoradiographs of follicles in early stages of atresia showed grains over the follicle cavity. In Text-fig. 3, the number of grains per $1600 \mu\text{m}^2$ over the cavity was plotted in relation to time. All grain numbers were corrected for the background counted outside the section. Each point on the curves represents a mean value of three counts. The grain number over the follicle cavity in Stage I atresia at any time except in 'immediate autoradiographs' was significantly higher than in non-atretic follicles and in follicles in Stage II atresia (Student's t test, $P < 0.01$).

DISCUSSION

The pyknotic nuclei

The appearance of pyknotic nuclei among granulosa cells has previously been described as a sign of atresia (Deane, 1952; Alfert, 1955; Burkl, 1963, 1965). The DNA-content in the pyknotic nuclei was determined by Alfert (1955) and found to be lower than in normal cells. This was thought to be due to the fact that some of the measurements were made on nuclear fragments rather than on intact nuclei. Burkl (1963) showed that degenerating cells in rat follicles contained small basiphilic cytoplasmic dots, containing DNA.

In the 'delayed autoradiographs', we found that not only the pyknotic nuclei in the degenerating cells, but also the basiphilic dots inside and outside the cells had become labelled. This indicates that the dots are recently released nuclear fragments. Their connection to the rest of the nucleus by thin bridges further suggests that they represent DNA from the cell itself (Pl. 1, Fig. 3).

The labelling over the cavity

The labelling over the follicular cavity in Stage I atresia in the 'delayed autoradiographs' was much higher than in the background, whereas the cavity in healthy follicles and in follicles in Stage II atresia never became labelled. The labelling in Stage I (Text-fig. 1) may be explained by the fact that the basement membrane was intact, and no leucocytes were present. The labelled nuclear material derived from disintegrated cells may therefore be retained in the cavity for some time. In Stage II, however, the basement membrane is broken down and leucocytes have invaded the follicle. This permits the labelled material to diffuse out of the follicle or to be phagocytosed by the leucocytes, which do show the presence of grains 2 and 3 days after pulse-labelling.

The labelling curves

The LI in a growing cell population remains constant during growth as

long as the labelled and unlabelled cells have the same proliferation rate. This is the case in follicles without signs of atresia (Text-fig. 2), which maintain a nearly constant LI during 6 days. A few of the healthy follicles have a very much higher LI on the 2nd and 3rd day. It appears that, in these apparently healthy follicles, two cell populations are present at the time of label injection: one which

TEXT-FIG. 4. Schematic representation of the process of follicular atresia and the normal development of follicles in the mouse. The large dots indicate the labelled cells and the small dots in the follicular cavities indicate the labelling over the cavities. The arrows show the direction in which the atretic processes or normal development might occur. The data for '0 days after [3H]thymidine' are those for follicles in autoradiographs prepared 1 hr after the injection.

continues to divide and is represented by the labelled cells, and another in which the proliferation rate is lower and which is represented by the unlabelled cells. In such follicles, the LI would increase with time after pulse-labelling.

In all atretic follicles, the LI varies with time; the LI in 'immediate autoradiographs' is much lower than that found in follicles without signs of atresia, but in the 'delayed autoradiographs' it exceeds the highest LI seen in non-atretic follicles. This increase in the LI in atretic follicles might also be

explained by a two-cell population theory. This would imply that the cells labelled at the time of injection would continue to divide preferentially. Many of them, however, will not contribute to the increasing LI as they become pyknotic within 24 hr.

Another mechanism must therefore be responsible for the increasing LI. A re-utilization of DNA released from dying cells, as shown with leucocytes (Bryant, 1962) and lymphocytes (Bryant, 1963), could account for this phenomenon. Results in the present experiments show that the labelled nuclear material is retained in the follicular fluid in Stage I atresia as seen in all 'delayed autoradiographs'. This nuclear material could be available for re-utilization by granulosa cells in S-phase, resulting in the increase in the LI. The failure of the LI to reach 100% is probably due to the fact that some of the cells are already in early stages of necrosis. As shown in the 'immediate autoradiographs' necrotic nuclei do not incorporate [^3H]thymidine.

The progressive nature of the three stages of atresia is primarily seen from their labelling curves, the shapes of which are similar though displaced in time by 1 day (Text-fig. 2). The fact that non-proliferating follicles in Stage II and Stage III are labelled in the 'delayed autoradiographs', and only here, indicates that they derive from follicles that previously were able to synthesize DNA, i.e. follicles without signs of atresia or follicles in Stage I atresia. This infers that follicles in Stage II and Stage III are continuous stages of atresia.

Text-figure 4 demonstrates schematically the way in which the follicles develop and change during the 6 days after pulse-labelling.

The follicles can be divided into two groups: (1) follicles with signs of atresia (Text-fig. 4, columns a to d), (2) follicles without signs of atresia (Text-fig. 4, columns e and f).

The vertical columns in the group of follicles with signs of atresia represent similar stages of atresia on different days after pulse-labelling. This schematically expresses how the LI changes with time after pulse-labelling, corresponding to the labelling curve of the follicle. The diagonal arrows indicate the direction in which the atretic process can occur.

In the group of follicles without signs of atresia, the progressive follicle growth is shown by vertical arrows (Column f). The change to follicles without signs of atresia, from which follicles in Stage I atresia derive (Column e), is indicated by horizontal arrows.

In order to see in which way a non-proliferating follicle in Stage II atresia gets its labelled cells 4 days after pulse-labelling (Text-fig. 4, a-4), the diagonal arrows should be followed backwards to pulse-labelling time (Text-fig. 4, e-0). At b-3, there is a follicle in Stage II atresia, with many labelled cells (60%). It is known, however, that very few cells in Stage II can synthesize DNA. The cells must therefore have incorporated the label during an earlier stage. If the arrow is followed backwards 1 day again from (b-3 to c-2), it leads to a follicle in Stage I, which also has about 60% labelled cells. The high LI can be explained by a re-utilization during the previous 24 hr of free labelled nuclear material in the follicular cavity (arrow to d-1). The follicle in d-1 is still in Stage I atresia but with a LI of only 25 and with a very well-labelled follicle cavity. The day before (e-0) it could have been a follicle without signs

of atresia. This backward calculation based on the four labelling curves gives the time it takes a follicle without signs of atresia to go through progressive stages of atresia. To reach Stage II and Stage III atresia takes about 3 and 4 days, respectively.

ACKNOWLEDGMENT

I thank Miss Gurli Rasmussen for technical assistance.

REFERENCES

- ALFERT, M. (1955) Changes in the staining capacity of nuclear components during cell degeneration. *Biol. Bull. mar. biol. Lab., Woods Hole*, **109**, 1.
- BANON, P., BRANDES, D. & FROST, J. K. (1964) Lysosomal enzymes in the rat ovary and endometrium during the estrous cycle. *Acta cytol.* **8**, 416.
- BRYANT, B. J. (1962) Reutilization of leucocyte DNA by cells of regenerating liver. *Expl Cell Res.* **27**, 70.
- BRYANT, B. J. (1963) Reutilization of lymphocyte DNA by cells of intestinal crypts and regenerating liver. *J. Cell Biol.* **18**, 515.
- BURKL, W. VON (1963) Zur Entstehung der Feulgen-positiven Einschlüsse in Liquor folliculi atresierenden Ovarialfollikel bei Ratten. *Mikroskopie*, **18**, 339.
- BURKL, W. VON (1965) Zur kausalen Genese der Follikelatresie. *Arch. Gynaek.* **200**, 689.
- DALMANE, A. P. (1967) Atresia of follicles in ovarium of mammals. *Archs Anat. Histol. Embryol.* **53**, 73.
- DEANE, H. W. (1952) Histochemical observations on the ovary and oviduct of the albino rat during the estrous cycle. *Am. J. Anat.* **91**, 363.
- GURAYA, S. S. & GREENWALD, G. S. (1964) A comparative histochemical study of interstitial tissue and follicular atresia in the mammalian ovary. *Anat. Rec.* **149**, 411.
- LOBEL, B. L., ROSENBAUM, R. M. & DEANE, H. W. (1961) Enzymic correlates of physiological regression of follicles and corpora lutea in ovaries of normal rats. *Endocrinology*, **68**, 232.