Tissue and Cell 42 (2010) 234–241

Contents lists available at ScienceDirect

Tissue and Cell

journal homepage: www.elsevier.com/locate/tice

Involvement of cell proliferation in the process of follicular atresia in the guinea pig

Wei Wang[a], Honglin Liu[a], Wei Ding[a,b], Yan Gong[a], Jingwei Chen[a], Reinhold J. Hutz[c], Dagan Mao[a], Fangxiong Shi[a,*]

[a] Laboratory of Animal Reproduction, College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, PR China
[b] Department of Animal Husbandry and Veterinary Medicine, Jiangsu Polytechnic College of Agriculture and Forestry, Jurong 212400, PR China
[c] Department of Biological Sciences, University of Wisconsin-Milwaukee, WI 53201, USA

ARTICLE INFO

Article history:
Received 20 January 2010
Received in revised form 21 April 2010
Accepted 27 April 2010
Available online 3 June 2010

Keywords:
Follicular atresia
Cell proliferation
Guinea pig

ABSTRACT

Cell morphology and proliferation was investigated in the atretic follicles during estrous cycles in the guinea pig. Ovarian samples on days 1, 4, 8, 12 and 16 of the estrous cycle in the guinea pig were taken in the morning for histologic staining with hematoxylin and eosin (HE), and immunohistochemical staining of the protein proliferating cell nuclear antigen (PCNA). The results indicated that the granulosa cells degenerated and eliminated first in atretic follicles, while the fibroblast-like cells appeared in the innermost layer of theca interna cells. When the fibroblast-like cells migrated to the antrum, they proliferated and formed a new tissue in peripheral to the zona pellucida of the oocyte. Our results also revealed that the orientation of the theca interna cell arrangement changed twice during the process of atresia, and the loose connective tissue in the antrum was critical for follicular atresia. Therefore, follicular atresia was not a simple process of cell death and elimination, but coexisted with cell proliferation. To our knowledge, we have for the first time confirmed cell proliferation and the presence of new tissue in atretic follicles in guinea pigs.

1. Introduction

A considerable amount of research has been performed on follicular atresia during the last decade, and the main work focused on cell death and elimination (Alonso-Pozos et al., 2003; Nezis et al., 2006; Santos et al., 2008). However, little was known about the cell proliferation in the process of follicular atresia. In our current paper, these phenomena were documented in detail in guinea pigs. In view of some morphologic characteristics described for the first time, several special terms were cited based on the similarity of structures and functions.

In the process of follicular atresia, the morphologic changes were obvious, and some polymorphic cells are differentiated from the innermost theca layer, which were described as "stellate or slender cells" (Kasuya, 1997), "fibroblastoid cells", "fibroblast-like cells" and "fibroblastoid-looking cells" (Logothetopoulos et al., 1995). In the present paper, the polymorphic cells were denominated as "fibroblast-like cells" (Wang et al., 2010).

It had been reported that a large numbers of fibroblast-like cells appeared in the antrum of atretic follicles, and documented as a "cellular network" (Kasuya, 1997). However, cell proliferation was not confirmed (Kasuya, 1997; Logothetopoulos et al., 1995). In the present study, the protein proliferating cell nuclear antigen (PCNA) was used as the proliferation marker (Bolton et al., 1994; Paunesku et al., 2001; Wildemann et al., 2003). PCNA – a cofactor of DNA polymerases that encircles DNA – orchestrates several of these functions by recruiting crucial players to the replication fork (Moldovan et al., 2007). Our results showed that cell proliferation did occur in atretic follicles. In addition, the term "cellular network" was not included in the changes within the follicular antrum. When a large number of fibroblast-like cells appeared, the antrum was not empty any more, and had very loose connections. This structure was described as "loose connective tissue" (Reed et al., 2009; Tzouvelekis et al., 2009; Weber, 1999; Wolf et al., 2009). Subsequently, with the proliferation of the differentiated cells, a new tissue formed in the antrum, which was described as "new tissue" (Leite et al., 2002). Although obvious changes occurred in the theca layers, the follicles were destined to degenerate. With the shrinking of follicular volume, atretic follicles lost their identifications (Logothetopoulos et al., 1995).

Cellular morphologic changes during follicular atresia were fully investigated in the present study using histology, and we confirmed

* Corresponding author.
E-mail addresses: weigrstar@yahoo.com.cn (W. Wang), fxshi@njau.edu.cn (F. Shi).

0040-8166/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tice.2010.04.006

follicular atresia was not a simple process of cell death and elimination, but coexisted with cell proliferation.

2. Materials and methods

2.1. Animals and sample collection

Twenty-five adult female guinea pigs (*Cavia porcellus*) of the Hartley strain were used at 5 months of age, with an initial weight of 400–700 g. They were housed 4 animals per cage, under controlled temperature at 23 ± 2 °C, and fed commercial food and tap water *ad libitum*. Estrous cycles were recorded by daily examination of vaginal smears whenever the vagina was open (Shi et al., 1999). The day of ovulation was estimated as the day when the maximal cornification was seen in the smear (Hutz et al., 1990; Lilley et al., 1997; Norris and Adams, 1979) and was designated as day 0 of the cycle. Days of the estrous cycle followed thereafter consecutively. We used only animals showing at least two consecutive 15–17-day cycles immediately prior to the experiment. The animals were sacrificed on days 1, 4, 8, 12, and 16 (5 animals per day) (Garris and Mitchell, 1979). Then the ovaries were collected immediately for experiments.

2.2. Reagents

PCNA monoclonal antibodies were purchased from Biogenix Co. (San Ramon, CA, USA, lot: Mu2060899); immunohistochemical kits (SABC methods) were purchased from Boshide Co. (Wuhan, Hubei, China, lot: SA1021); 3,3′-diaminobenzidine tetrachloride (DAB) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were purchased reagent grade.

2.3. Histological and immunohistological studies

Ovaries were fixed in 4% paraformaldehyde at room temperature for 24 h, embedded in paraffin, sectioned serially at 10 μm and stained with hematoxylin and eosin (HE). These sections were analyzed for the morphologic changes indicative of atretic follicles. To examine the proliferating cells in the atretic follicles, immunohistochemical staining was performed using monoclonal antibodies to PCNA with the streptavidin–biotin-complex (SABC) method. A few sections were picked randomly from the serial sections. Sections were mounted on slides coated with APES and which were dried for 24 h at 37 °C. The antibodies were diluted 1:500 in PBS containing 1% bovine serum albumin (BSA), and the sections were incubated overnight at 4 °C with primary antibodies. The specific protein immunoreactivity was visualized by 0.05% DAB in 10 mM PBS-buffered saline containing 0.01% H_2O_2 for 2–5 min, and counterstained with hematoxylin. A negative control was a primary antibody blank, and relative levels of immunostaining were evaluated and repeated at least four times. The percent area of follicular cells staining positive for PCNA by more than 50% were considered strongly positive, between 10 and 50% were considered positive, and less than 10% were considered to be negative. The images were captured in a Nikon 80i (Nikon, Tokyo, Japan). Three areas at 50 μm × 50 μm were selected randomly for evaluation within each follicle, and at least six follicles were evaluated for each stage of follicular atresia.

2.4. Evaluation of follicular atresia

Follicles were considered as round shape in the sections, and cellular arrangement was assigned in centripetal or tangent direction. The tangent direction was perpendicular to centripetal direction. Follicular diameter, thickness of theca interna cells, and the ratio of L1 (diameters in centripetal direction) to L2 (diameters in tangent direction) in the nuclei of theca interna cells were measured for evaluating the follicular atresia. It was widely accepted that the follicular growth of guinea pigs was biphasic (Bland, 1980; Fortune, 1994; Hutz et al., 1990; Shi et al., 1999). In the current study, follicles on days 16, 1 and 4 were investigated, which constituted the second wave of follicular growth and atresia. Only those follicles with the largest cross-sectional area were measured, and we ensured that each follicle was measured only once. The sample size of each group was at least twenty. Microscopic measurements were made with a Nikon 80i (Nikon, Tokyo, Japan).

Follicular diameter was taken as the mean of the largest diameter and the diameter perpendicular to the midpoint (Bland, 1980). Thickness of theca interna cells was measured at three different positions, and the mean was used for statistical analyses. The ratio of L1 to L2 was taken to denote the arrangement changes in the nuclei of theca interna cells, and the migrated fibroblast-like cells which had very thin shapes were excluded. At least three areas at 50 μm × 50 μm were measured in each follicle, and the mean was used for statistical analyses. Therefore, the ratio more than or less than 1.0, respectively, represented the arrangement of theca interna cells in centripetal or tangent direction.

3. Results

3.1. Follicular atresia in normal estrous cycles

On day 1 after ovulation, there were very few healthy antral follicles in the ovaries, and the new corpora lutea were observed. A mass of dead granulosa cells separated from the granulosa layers and scattered in the antrum. Furthermore, in some atretic follicles where granulosa cells were eliminated, fibroblast-like cells appeared in the theca interna cells.

On day 4, the most atretic follicles were shrunken, and the follicular antrum was filled with fibroblast-like cells. Some atretic follicles exhibited a very small volume and the fibroblast-like cells disappeared.

On days 8 and 12, a group of large antral follicles appeared. Many of these acquired large population of granulosa cells. However, the antral follicles were often in early stages of atresia.

On day 16, antral follicles were further developed. Ovaries at this time contained 0–3 unruptured preovulatory follicles, which had diameters at least 700 μm. Although many follicles showed large volumes, there was a mass of desquamated granulosa cells and were distributed along the follicular cavity. At this time, it was obvious that the intensity of the follicular atresia was greater than on either day 8 or 12.

3.2. Cell elimination in follicular atresia

Based on the morphologic observations (Figs. 1 and 2), there were continuous changes in the process of follicle atresia in guinea pigs. The most remarkable characteristics included the elimination of granulosa cells and the formation of new tissue. Moreover, it seemed that the fibroblast-like cells played a central role in new tissue formation.

In healthy follicles, granulosa cells were arranged neatly (Fig. 1A and Fig. 2A). When the atretic process began, connections in the granulosa layers became loose, and the columnar granulosa cells became round (Fig. 2B), with many pyknotic cells and apoptosis bodies were observed (Fig. 2C). With the massive elimination of granulosa cells, granulosa layers disintegrated gradually, and dead granulosa cells were shed into the follicular antrum (Fig. 1B). At the same time, the fibroblast-like cells appeared in the innermost layer of theca cells (Fig. 2D).