Enzyme Histochemical Observations on the Ovarian Small Follicles in Relation to the Mechanism of Atresia in Laying Hens (*Gallus domesticus*)

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In order to examine the mechanism of atresia in the small follicles of laying hens, changes of three enzyme activities, acid phosphatase, 3β -hydroxysteroid dehydrogenase (3β -HSD) and glucose-6-phosphate dehydrogenase (G-6-PD), during atresia were analyzed histochemically. Acid phosphatase activity in the follicular wall increased along with the tissue degradation in the process of atresia. High activities of 3β -HSD and G-6-PD which were observed only in the thecal interstitial cells of small follicles did not decrease at the early stage of atresia, but decreased as atresia proceeded. It is suggested that lysosomal enzymes may be involved in the tissue degradation during atresia. It is perhaps premature to conclude that the decrease of the production activities of steroid hormones, whose synthesis depends on 3β -HSD and G-6-PD, in individual follicles is involved in the induction of atresia.

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Key words : ovarian small follicle, atresia, steroid hormones, lysosomal enzymes

Introduction

The ovarian follicles of the hen can be divided into two distinct populations, the large yellow follicle group (larger than about 8 mm in diameter), and the small white follicle group (smaller than about 8 mm)¹⁾. In the mature laying hens, atresia which is rarely observed in the former group occurs commonly in the majority of the follicles of the latter group²⁾. The atresia of small follicles may be closely related to the egg production rate of a bird; e. g., the hierarchy of large yellow follicles may be determined by the loss of small follicles (1-8 mm in diameter) through atresia²⁾, and the high incidence of atresia in these follicles may eventually lead to the decrease of the ovulation rate.

Histological studies have revealed that the atresia of small white follicles is characterized by the invasion of granulosa and theca cells into the follicle¹⁾, but the mechanism underlying the induction of atresia has not been fully elucidated. Recently, it has been reported that progesterone receptors were present in the granulosa and theca cells of the small follicles of laying hens³⁾. The decrease of the thecal aromatase activity, which is involved in estrogen synthesis, during the atresia of the small follicles has been demonstrated in an enzyme assay study⁴⁾. Based on

these findings, it is estimated that certain functions of the granulosa and theca cells of the small follicles may be controlled by steroid hormones, and that the decrease of the concentration of steroid hormones and/or their production activities within individual follicles may lead to atresia. On the other hand, the role of lysosomal enzymes in the tissue degradation has been suggested in atretic yellow follicles⁵⁾, but the factors involved in the tissue degradation during atresia of small follicles have not been elucidated. This study was, therefore, undertaken to analyze the mechanism of atresia of small follicles by studying histochemically the changes of the activities of lysosomal enzymes and of the enzymes involved in steroidogenesis during the process of atresia. The following enzymes were examined: 1) acid phosphatase, a marker enzyme of lysosomes; 2) 3 β -hydroxysteroid dehydrogenase (3 β -HSD), an enzyme necessary for the synthesis of progesterone from pregnenolone and testosterone from 5-androsterone-3 β , 17 β -diol⁶⁾; 3) glucose-6-phosphate dehydrogenase (G-6-PD), an enzyme which provides the NADPH required for steroid synthesis⁷⁾.

Materials and Methods

A total of eight White Leghorn hens weighing 1.5 to 1.8 kg, 10 to 15 months-old, and laying four eggs or more in a sequence were used in this study. They were maintained in individual cages on a 14 h light regimen and provided with food and water *ad libitum*. Hens were killed 2 to 5 hrs after oviposition, and their ovarian stroma tissue was quickly frozen in an isopentane and solid CO₂ mixture, sectioned by use of a cryostat (10 μ m in thickness), and dried on a glass slide. Some of the sections were fixed in a 10% formalin solution and stained with hematoxylin and eosin. The other sections were used to examine the activities of the enzymes by the following methods: *acid phosphatase activity*, sections were fixed in cold acetone (4°C), treated by the method of BARKA and ANDERSON⁸⁾ and counter stained with hematoxylin; *3 β -HSD activity*, sections were fixed in cold acetone and treated by the method of BLAHA and LEAVITT⁹⁾ using pregnenolone (Sigma Chem. Co.) as a substrate; *G-6-PD activity*, unfixed sections were treated by the method of RUDDPH and KLEIN⁷⁾, and fixed with 10% formalin. In all cases, control sections were prepared by incubation with the reaction mixtures in omitting the respective substrates, and appropriate results were obtained. Observations were made on the follicles about 0.5-1 mm in diameter.

Results

The follicles smaller than about 1 mm in diameter were located in the connective tissue of the ovarian cortical stroma. The normal follicular wall was composed of the inner granulosa layer and outer thecal layer (Fig. 1). The granulosa layer was one or two celled thick. The thecal layer consisted of dense connective tissue containing fibroblasts and interstitial cells. At the early stage of atresia, the disintegration of the granulosa layer and a slight loosening of the thecal tissue were observed (Fig. 2). The granulosa cells detached from the follicular wall showed a degraded appearance. The late stage of atresia was characterized by the invasion of a large number of cells

from the granulosa and thecal layers (Fig. 3).

No reaction products exhibiting acid phosphatase activity could be found in the normal follicular wall except for a small amount of products in the granulosa cells (Fig. 4). The reaction was more pronounced in the follicular wall at the early stage of atresia. Granulosa cells including those attached to and detached from the wall and some fibroblasts in the thecal tissue contained dense reaction products (Fig. 5). At the late stage of atresia, a large number of reaction products were observed in most of the cells forming the follicular wall and in invading cells (Fig. 6).

The dense formazan deposits showing 3 β -HSD activity in the normal follicles were observed only in the thecal interstitial cells, but not in any other types of cells including the granulosa cells (Fig. 7). Differences could not be found in the amount of reaction products between normal follicle and early stage of atresia (Fig. 8). However, along with the progression of the disintegration of the follicular tissues, the amount of reaction products decreased gradually (Fig. 9). Cells in the wall of follicles at the late stage of atresia did not contain reaction products.

In the normal follicles, markedly dense formazan deposits showing G-6-PD activity were observed in the thecal interstitial cells, in contrast to the very small amount of deposits in the other cells such as granulosa cells and thecal fibroblasts (Fig. 10). Difference in the amount of formazan deposits could not be observed even when evidence of early atretic change was found (Fig. 11). The reaction products could not be observed in follicles at the late stage of atresia except in some scattered cells showing a marked decrease in the reaction (Fig. 12) as compared with the normal interstitial cells.

Discussion

The invasion of cells from the granulosa and thecal layers was observed during the process of atresia of small follicles as reported previously¹⁾. The first structural changes of this process consisted of the disintegration of the granulosa layer, followed by the degradation of the thecal tissues. The increase of the acid phosphatase activity, which is a marker of lysosomal enzyme activities, in the follicular wall was closely associated with these structural changes. The activity increased in the disintegrated granulosa cells of the follicle at the early stage of atresia and in invading cells at the late stage of atresia. The correlation between the structural changes of the follicular tissues and the increase of acid phosphatase activity strongly suggests that lysosomal enzymes may be involved in the degradation of the follicular tissues during the process of atresia. In the larger follicles, in which atresia is characterized by the bursting of the follicular wall, it is also assumed that lysosomal enzymes may be involved in the atretic changes of the follicular wall⁵⁾.

On the other hand, it is considered that in small follicles of laying hens certain functions of the granulosa and theca cells may be controlled by steroid hormones, since progesterone receptors are present in these cells and the number of these receptors increased by the exogenous administration of estrogen³⁾. The present histochemical study revealed the presence of two enzyme activities, 3 β -HSD and G-6-PD, which are

Fig. 1. Section of a normal small follicle. Follicular wall is composed of the granulosa layer (G) and thecal layer (T). Arrow heads indicate the interstitial cells. HE staining. Scale bar=100 μ m.

Fig. 2. Section of a follicle at the early stage of atresia. Note the disintegration of the granulosa layer (arrow). HE staining. Scale bar=100 μ m.

Fig. 3. Section of a follicle at the late stage of atresia. Invasion of cells from the granulosa and thecal layers can be seen. HE staining. Scale bar=100 μ m.

Fig. 4. Photomicrograph showing acid phosphatase activity in a normal small follicle. No dense reaction products are observed. Scale bar=50 μ m.

Fig. 5. Photomicrograph showing acid phosphatase activity in a follicle at the early stage of atresia. Reaction products can be seen in the granulosa cells (arrow heads) and in some of the fibroblasts in the theca (arrows). Scale bar=50 μ m.

Fig. 6. Photomicrograph showing acid phosphatase activity in a follicle at the late stage of atresia. Dense reaction products are located in the invaded and degraded cells (arrows). Scale bar=50 μ m.

Fig. 7. Photomicrograph showing 3β -hydroxysteroid dehydrogenase (3β -HSD) activity in a normal follicle. Dense formazan deposits (blue in color) are present in the thecal interstitial cells. Scale bar = $100\ \mu\text{m}$.

Fig. 8. Photomicrograph showing 3β -HSD activity in a follicle at the early stage of atresia. Distribution and amount of formazan deposits in the interstitial cells are similar to those in a normal follicle. Scale bar = $100\ \mu\text{m}$.

Fig. 9. Photomicrograph showing 3β -HSD activity in a follicle at the late stage of atresia. Formazan deposits cannot be observed. Arrows show non-formazan deposits (brown in color). Scale bar = $100\ \mu\text{m}$.

Fig. 10. Photomicrograph showing glucose-6-phosphate dehydrogenase (G-6-PD) activity in a normal follicle. Thecal interstitial cells contain dense formazan deposits. Scale bar = $100\ \mu\text{m}$.

Fig. 11. Photomicrograph showing G-6-PD activity in a follicle at the early stage of atresia. Distribution of formazan deposits does not differ from that in a normal follicle (Fig. 10). Scale bar = $100\ \mu\text{m}$.

Fig. 12. Photomicrograph showing G-6-PD activity in a follicle at the late stage of atresia. Formazan deposits are not observed except for small deposits in scattered cells (arrows). Scale bar = $100\ \mu\text{m}$.

involved in steroid hormone synthesis, in normal and atretic follicles. The activity of 3 β -HSD was restricted to the thecal interstitial cells and was not observed in other types of cells in normal follicles as reported by previous workers¹⁰⁾, and the intensity of the activity of G-6-PD followed a similar distribution. These findings indicate that the thecal interstitial cells have the ability to produce steroid hormones such as progesterone whose synthesis depends on these enzymes. The activity of both 3 β -HSD and G-6-PD did not decrease at the early stage of atresia, but decreased as atresia proceeded. Therefore, it cannot be concluded that the decrease of the production activity of steroid hormones, whose synthesis depends on the activity of these enzymes, within individual small follicles is involved in the induction of atresia of them. It appears that the decrease of the concentration and/or production activity of such steroid hormones in a follicle occurs as a result of the degradation of the follicular tissues.

Although it is considered that small follicles produce estrogen^{11,12)}, the presence of 3 β -HSD and G-6-PD activities does not imply that these enzymes are involved in production of estrogens. An enzyme assay study has shown that the thecal aromatase activity which is involved in estrogen production is less strong in atretic small follicles (about 1-8 mm in diameter) than in normal ones⁴⁾. More detailed histochemical and histological studies are required to determine the relationships between the follicular atretic changes and estrogen production activity.

Although the significance of receptors must be examined to determine the physiological roles of hormones, the relationships between atresia and receptors of hormones including gonadotrophins and steroid hormones remain unknown. The invasion of cells from the follicular tissues indicates that close relationships may exist between the oocyte degeneration and incidence of atresia. Further investigations are necessary to clarify these relationships.

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産卵鶏における閉鎖白色卵胞の酵素組織学的観察

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産卵鶏における白色卵胞の閉鎖機構を検討する目的で、卵胞内の酸性ホスファターゼ、ステロイド- 3β -水酸基・脱水素酵素 (3β -HSD) およびグルコース-6-リン酸脱水素酵素 (G-6-PD) 活性の閉鎖過程における変化を酵素組織学的に調べた。酸性ホスファターゼ活性は、正常卵胞組織内で低かったが、閉鎖に伴う変性組織内で高かった。正常卵胞内の 3β -HSD および G-6-PD は間質細胞内で高い活性を示した。両酵素の活性は、閉鎖初期卵胞では正常卵胞との差を示さなかったが、組織変性

の進行とともに消失した。以上の結果から、卵胞閉鎖過程における組織変性にライソゾーム酵素が関与するものと考えられた。一方、 3β -HSD および G-6-PD 活性から判断する限り、これらの酵素によって産生されるステロイドホルモンの減少と閉鎖開始との関連は不明であった。

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