Changes in the localization of MHC class II positive cells in hen ovarian follicles during the processes of follicular growth, postovulatory regression and atresia

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The aim of this study was to determine the changes in the population of major histocompatibility complex class II positive (MHC-II⁺) cells in ovarian follicles during the processes of follicular growth, postovulatory regression and follicular atresia in hens. Cryostat sections of ovarian stroma containing cortical follicles, small white follicles, the largest (F₁) and third largest (F₃) preovulatory follicles, postovulatory and atretic follicles of laying hens were prepared. The sections were immunostained for MHC-II molecules using mouse anti-chicken MHC-II monoclonal antibody and positive cells were counted using a computer-assisted image analyser under a light microscope. MHC-II⁺ cells were localized in the theca layer of normally growing follicles including cortical follicles, small white follicles and F₃ and F₁ preovulatory

follicles, whereas they were found in both the theca and granulosa layers in postovulatory and atretic follicles. The frequency of MHC-II⁺ cells in the theca layer was significantly increased during follicular growth from cortical follicles to F₃ preovulatory follicles. Although the population of MHC-II⁺ cells did not differ between F₃ and F₁ preovulatory follicles, it increased significantly in postovulatory follicles ($P < 0.01$). The population of MHC-II⁺ cells was significantly greater in the theca layer of atretic follicles than in non-atretic follicles ($P < 0.01$). These results indicate that the antigen-presenting function via MHC-II increases in association with follicular growth. A marked increase in MHC-II⁺ cells indicates that these cells may be involved in regression of postovulatory and atretic follicular tissues.

Introduction

Major histocompatibility complex class II (MHC-II) molecules are cell surface glycoproteins that are found in monocytes, macrophages and activated B and T cells (Vainio *et al.*, 1987; Bourlet *et al.*, 1988). An immune response against an antigenic agent requires a series of cellular interactions. One of these events is the processing and presentation of antigens by antigen-presenting cells containing MHC-II molecules (Maccubbin and Schierman, 1986; Vainio *et al.*, 1987). MHC-II molecules present antigens to the helper subset of T cells (CD4 T cells) and, thus, play central roles in the initiation of immune reactions (Benacerraf, 1981; Vainio *et al.*, 1988; Arstila, 1994).

Immunocompetent cells including MHC-II positive (MHC-II⁺) cells in the ovary could play important roles in the protection of ovarian tissues from infection and prevention of disease transmission to eggs. Recently, it was found that MHC-II⁺ cells are present in the ovarian stroma and theca layer of cortical follicles (Barua and Yoshimura, 1999). However, it is not known whether these immunocompetent cells play a role in the local modulation of ovarian functions in hens.

In addition to disease resistance, MHC-II antigens have been reported to be involved in the modulation of ovarian functions, such as regression of corpora lutea (Pate, 1995), degradation of atretic follicular tissues (Fujiwara *et al.*, 1993), premature ovarian failure (Hill and Anderson, 1990) and certain autoimmune disorders (Bottazzo *et al.*, 1983; Coulam and Stern, 1991) in mammals. Chicken MHC-II molecules are similar in structure and function to those of mammals (Vainio *et al.*, 1987; Kaufman, 1999). Therefore, it is possible that chicken MHC-II molecules, as reported in mammals, play important roles not only in protection from infection but also in regulation of follicular function. However, there are no reports about whether the localization of MHC-II⁺ cells changes with respect to follicular growth, postovulatory regression and atresia of follicles. The aim of the present study was to determine whether the localization of MHC-II⁺ cells in follicular tissues changes during follicular development, postovulatory regression and atresia.

Materials and Methods

Birds

White Leghorn laying hens aged approximately 450 days and laying more than five eggs in a sequence were used

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($n = 5$). The hens were kept in individual cages under a light regimen of 14 h light:10 h dark, and feed and water were available *ad libitum*.

Tissue preparation

Birds were killed by decapitation just after oviposition (approximately 30 min before the expected time of ovulation) (Warren and Scott, 1935). Ovarian stroma in which cortical follicles were embedded, normal and atretic small white follicles approximately 3–4 mm in diameter, the third largest and largest preovulatory follicles (F_3 and F_1 , respectively), and postovulatory follicles of day 1 and day 3 after ovulation were collected. Atretic small white follicles with deformed condition were also collected. All tissues were embedded in OCT compound and snap frozen in a mixture of isopentane and solid carbon dioxide. Cryostat sections (15 μm thickness) were prepared and air dried on slides treated with 3-aminopropyl-triethoxysilane as described by Van Prooijen-Knegt *et al.* (1982). The sections were then fixed with cold acetone and methanol for 10 min each.

Antibodies

Mouse monoclonal anti-chicken MHC-II antibody (Veromaa *et al.*, 1988) was used as the primary antibody. Immunodetection kit S-HRP (Seikagaku Kogyo Co, Tokyo) was used for detection of immunoprecipitates of primary antibodies.

Immunostaining

Sections were washed with PBS for 15 min (3×5 min) and incubated with 1% (v/v) normal goat serum for 15 min to block non-specific binding of antibodies. Sections were incubated overnight with mouse anti-chicken MHC-II antibody diluted with PBS containing 0.5% (w/v) BSA at a dilution of 1:100, followed by washing with PBS for 15 min (3×5 min). Immunoreactions of the first antibody on the sections were detected by S-HRP immunostaining kit according to the manufacturer's instructions. In brief, the sections were incubated with the biotinylated secondary antibody and with avidin–biotin–peroxidase complex for 1 h each. Finally, immunoprecipitates were visualized by incubation with a mixture of 0.02% (w/v) 3',3'-diaminobenzidine–tetrahydrochloride (Sigma, St Louis, MO) and 0.001% (w/v) H_2O_2 in 0.05 mol Tris–HCl I⁻¹ (pH 7.6). The sections were counterstained with haematoxylin after immunostaining, dehydrated and covered. Control staining was carried out simultaneously in which the first antibody was replaced with normal mouse IgG. No specific staining was found in the control slides.

Counting of MHC-II⁺ cells

The sections were examined under a light microscope with image analysis software (Image-Pro Plus, Media

Cybernetics, Silver Spring, MD). The numbers of immunopositive cells were obtained by observing three different regions of the theca (approximately $0.5\text{--}3.0 \times 10^4 \mu\text{m}^2$ tissue) as described by Barua *et al.* (1998a). The number of cells was calculated to be the number of cells in $1 \times 10^4 \mu\text{m}^2$ tissue. The mean of the three counts was expressed as the number of cells in $1 \times 10^4 \mu\text{m}^2$ area in one tissue of a bird.

Statistical analysis

The significance of differences in the number of positive cells in $1 \times 10^4 \mu\text{m}^2$ area was determined by one-way ANOVA (Snedecor and Cochran, 1967), followed by Duncan's multiple range test (Duncan, 1955). Student's *t* tests were used for the analysis of positive cells in atretic follicles and differences between the means were considered significant at $P < 0.01$.

Results

Changes in the frequency of MHC-II⁺ cells during follicular growth and postovulatory regression

The cortical follicles were embedded in the ovarian stroma and the small white follicles and F_3 and F_1 preovulatory follicles protruded from the ovarian surface. The wall of cortical follicles, small white follicles and F_3 and F_1 preovulatory follicles consisted of the theca and granulosa layers. In the day 1 postovulatory follicles the theca layer became contracted with partial detachment of granulosa layer and in the day 3 postovulatory follicles the theca and granulosa layers became more regressed. MHC-II⁺ cells were localized in the theca layer but not in the granulosa layer of normal cortical follicles, small white follicles and F_3 and F_1 preovulatory follicles, whereas they were localized in both the theca and granulosa layers in postovulatory follicles (Fig. 1 a–f). Some thecal fibroblast-like cells in these prehierarchical and hierarchical follicles also stained positive for MHC-II. These results were observed equally in five repeated observations. The frequency of MHC-II⁺ cells in the theca layer of normal cortical follicles was 4.5 cells in $1 \times 10^4 \mu\text{m}^2$ and it increased significantly to 9.9 cells in $1 \times 10^4 \mu\text{m}^2$ in small white follicles ($P < 0.01$) (Fig. 2). The frequency of MHC-II⁺ cells increased significantly in the theca layer of F_3 and F_1 preovulatory follicles compared with cortical follicles and small white follicles ($P < 0.01$). The population of MHC-II⁺ cells in the theca layer of F_3 preovulatory follicles was 15.3 cells in $1 \times 10^4 \mu\text{m}^2$ and no significant difference was observed in the frequency of MHC-II⁺ cells between F_3 and F_1 preovulatory follicles. The frequency of MHC-II⁺ cells in the theca layer of postovulatory follicles was significantly greater than in preovulatory follicles. However, no significant difference was observed in the population of MHC-II⁺ cells between day 1 and day 3 postovulatory follicles (Fig. 2).

Fig. 1. Sections of chicken ovarian follicles immunostained for major histocompatibility complex class II (MHC-II). (a) Ovarian stroma containing cortical follicle. (b) Small white follicle. (c) Third largest preovulatory follicle. (d) Largest preovulatory follicle. (e) Postovulatory follicle on day 1 after ovulation. (f) Postovulatory follicle on day 3 after ovulation. Thecal fibroblast-like cells stained positive for MHC-II in all follicles. In the granulosa layer, positive cells are seen in the postovulatory follicles only. Arrows indicate examples of MHC class II positive cells. G: granulosa layer; LCT: loose connective tissue coat; O: oocyte; S: ovarian stroma; T: theca layer; Y: yolk. Scale bars represent 50 μm .

Changes in the frequency of MHC-II⁺ cells during follicular atresia

Atretic cortical follicles embedded in the stroma were observed occasionally in the ovarian sections, whereas atretic small white follicles protruded from the ovarian surface. Granulosa cells were detached from the theca layer

Fig. 2. Changes in the population of major histocompatibility complex class II positive (MHC-II⁺) cells in ovarian follicles during follicular growth and postovulatory regression. CF: cortical follicle; SWF: small white follicle; F₃: third largest preovulatory follicle; F₁: largest preovulatory follicle; RF₁: postovulatory follicle on day 1 after ovulation; RF₃: postovulatory follicle on day 3 after ovulation. Values are mean $\pm$ SEM ($n = 5$ birds in each group). ^{abcd}Columns with different letters are significantly different ($P < 0.01$).

Fig. 3. Sections of atretic follicles of laying hens immunostained for major histocompatibility complex class II (MHC-II⁺) positive cells. (a) Atretic cortical follicle and (b) atretic small white follicle. The granulosa layers are disintegrated, detached from the thecal layer and invaded into the follicular lumen in both categories of atretic follicle. MHC-II⁺ cells are observed in the granulosa and theca layers as well as in the follicular lumen. O: oocyte; G: granulosa layer/cell; T: theca layer. Arrows indicate examples of MHC-II⁺ cells. Scale bars represent 50 µm.

Fig. 4. Frequency of major histocompatibility complex class II positive (MHC-II⁺) cells in normal (□) and atretic (■) follicles in chickens. Values are mean ± SEM ($n = 5$ birds in each group). *Significantly different from corresponding normal value ($P < 0.01$).

and invaded the follicular lumen in both categories of atretic follicles. MHC-II⁺ cells were found in the granulosa and theca layers as well as in the follicular lumen of atretic follicles (Fig. 3a,b). The frequencies of MHC-II⁺ cells in the theca layer were significantly greater in both the atretic cortical follicles and small white follicles (approximately 15 cells and 19 cells in $1 \times 10^4 \mu\text{m}^2$, respectively) compared with normal follicles ($P < 0.01$) (Fig. 4).

Discussion

This is the first report in which the changes in the localization of MHC-II⁺ cells in hen ovarian follicles have been examined with respect to follicular events. The major findings were: (i) MHC-II⁺ cells were localized in the theca layer of normally growing follicles, but in both the theca and granulosa layers of postovulatory and atretic follicles; (ii) the frequency of thecal MHC-II⁺ cells was significantly increased in association with follicular growth from cortical follicles to F₃ preovulatory follicles; however, no difference in their frequencies was observed between F₃ and F₁ preovulatory follicles; and (iii) an increased population of MHC-II⁺ cells was observed in the theca layers of postovulatory and atretic follicles.

In a previous study, we reported the presence of MHC-II⁺ cells in some of the thecal fibroblast-like cells in the cortical follicles (Barua and Yoshimura, 1999). In the present study, it was found that some fibroblast-like cells of the theca layer expressed MHC-II antigen not only in prehierarchical but also in hierarchical follicles. Carbon particles that were injected i.v. into laying hens were internalized by the fibroblasts in the theca interna of preovulatory follicles, indicating that these cells may be able to phagocytose foreign antigens (Yoshimura and Okamoto, 1998). Therefore, the antigen presenting ability of the thecal fibroblast-like cells may play a significant role in the protection of growing yolk from pathogenic organisms passing through the follicular wall.

Presl and Bukovsky (1986) identified MHC-II⁺ cells in the theca layer of normal growing follicles in rats and suggested that these immunocompetent cells might be involved in normal follicular development. In the present study, the population of MHC-II⁺ cells increased during follicular growth from cortical follicles to F₃ preovulatory follicles. Therefore, it is possible that expression of MHC-II⁺ cells is enhanced with follicular development in both chickens and mammals. In a previous study, we observed that treatment of immature birds with oestrogen enhanced expression of MHC-II antigens in the ovarian stroma and theca of cortical follicles (Barua and Yoshimura, 1999). Oestrogen is produced by aromatase cells in the theca externa of smaller follicles and there is a linear decrease in oestrogen production with the lowest production in F₁ preovulatory follicles (Bahr *et al.*, 1983; Bahr, 1991; Johnson, 1996). Therefore, oestrogen may be one of the local regulators to increase MHC-II⁺ cell frequency in the theca of growing smaller yellow follicles. During the later stage of follicular growth, for example from F₃ to F₁ preovulatory follicles, oestrogen production is low and the thecal MHC-II⁺ cell frequency is not changed significantly.

The finding that there was no significant difference in the thecal MHC-II⁺ cell population between F₃ and F₁ preovulatory follicles collected approximately 30 min before ovulation indicates that there is no increase in MHC-II⁺ cells of these follicles with ovulation. In the present study, the number of thecal MHC-II⁺ cells in the day 1 and day 3

postovulatory follicles was greater than in F₁ and F₃ preovulatory follicles, and MHC-II⁺ cells appeared in the granulosa layer of postovulatory follicles but not preovulatory follicles. In mammals, there are reports that MHC-II⁺ antigen is expressed in the cells of corpora lutea that had undergone regression (Kenny *et al.*, 1991; Pate, 1995). Thus, it may be proposed that MHC-II⁺ cells play a role in the regression of postovulatory follicles in hen ovaries.

In atretic follicles, MHC-II⁺ cells were localized in both the granulosa and theca layers, and their frequencies in the theca were significantly greater than in normal follicles. MHC-II protein was reported to be present on granulosa cells of atretic cortical follicles in human ovaries (Fujiwara *et al.*, 1993). Previously, we reported that macrophages, which express MHC-II⁺, infiltrate the atretic follicles (Barua *et al.*, 1998b). These macrophages were localized in the thecal connective tissue and granulosa layer or follicular lumen. The region where MHC-II⁺ cells were localized in the atretic follicles was identical to that in which macrophages were found. Therefore, many of the MHC-II⁺ cells in the theca and granulosa layers are likely to be macrophages, and they may play a role in the removal of atretic bodies, as suggested in our previous study (Barua *et al.*, 1998b).

In conclusion, it was found that the population of MHC-II⁺ cells increased with follicular growth as well as postovulatory regression and follicular atresia. These cells may play significant roles not only in the removal of infectious or antigenic agents in the growing follicles but also in tissue regression by apoptosis of postovulatory and atretic follicles.

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