Changes in the Localization of Immunoreactive Avian β -Defensin-12 in Ovarian Follicles during Follicular Growth and in Response to Lipopolysaccharide

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The aim of this study was to identify the avian β -defensin-12 (Av β D-12) protein in the ovarian follicles and to determine the changes in its localization during follicular growth and in response to lipopolysaccharide (LPS). Anti-Av β D-12 polyclonal antibody was raised using synthetic peptide. White Leghorn laying hens were i.v. injected with or without LPS (1 mg/kg BW). White follicles and yellow follicles (the largest and the third largest follicles; F₁ and F₃, respectively) were collected before and after 12 or 24 h of LPS injection ($n=3$ in each group). Immunocytochemistry using an avidin-biotin-peroxidase complex method was performed using their paraffin sections. The immunoreactive Av β D-12 (irAv β D-12) was not found in the white follicles. The irAv β D-12 was localized in the capillary-associated cells in the outer theca interna, granulosa cells and perivitelline layer of yellow follicles. The irAv β D-12 in the theca interna and granulosa layers of yellow follicles was decreased by 12 h and disappeared by 24 h of LPS injection. Perivitelline layer was kept positive for irAv β D-12 even after 24 h of LPS injection. No significant difference was found in the distribution of irAv β D-12 between F₃ and F₁ for the above results. No immunoreaction products were observed in the sections incubated with absorbed antibody of Av β D-12 instead of first antibody (control staining). These results suggest that the theca interna, granulosa and perivitelline layers contain Av β D-12. Its amount is likely increased with follicular growth from a white follicle to a yellow follicle, whereas the protein may be released from the cells by LPS stimulation, probably as a host defense response.

Key words: avian β -defensin-12, follicular growth, laying hen, lipopolysaccharide, ovarian follicle

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Introduction

The ovary of laying hen consists of cortical follicles embedded in the stroma, white follicles, preovulatory yellow follicles and postovulatory follicles protruded from the stroma (Johnson, 1996). Follicular wall consists of the superficial tissue, theca, granulosa layer, and perivitelline membrane. Pathogenic microorganisms such as *Salmonella enteritidis* may infect ovarian tissues and can be transmitted to eggs (Keller *et al.*, 1995; Thiagarajan *et al.*, 1996; Takata *et al.*, 2003; Barua and Yoshimura, 2004). Thus, the ovarian immunity is essential to protect the ovarian tissues from infection and production of hygienic eggs. With the growth of the follicles, the theca interna and externa develop in thickness and blood supply increases (Scanes *et al.*, 1982; Wolfenson *et al.*, 1982). The steroidogenic profiles also change during the growth, namely the estrogen production is higher in smaller follicles, however, that of progesterone is higher in larger

follicles (Gilbert, 1979; Bahr *et al.*, 1983). With these structural and functional changes, immune functions in the follicles may also be changed.

Avian β -defensins (Av β Ds) are chicken antimicrobial peptides that play a role in the innate immune response (Yang *et al.*, 2002). Gene expression of thirteen Av β Ds (Av β D1-13) that are effective against various organisms including Gram-positive and Gram-negative bacteria, fungi, yeast and some viruses have been reported in different tissues (Evans *et al.*, 1995; Sugiarto and Yu, 2004; Xiao *et al.*, 2004; Lynn *et al.*, 2007). The mRNA expression of Av β D-12 was found in both theca and granulosa layers of the ovarian follicles and the expression in those tissues was constant during follicular growth (Subedi *et al.*, 2007a). In response to lipopolysaccharide (LPS) injection, the mRNA expression of Av β D-12 was increased in the theca, however decreased in the granulosa layer (Subedi *et al.*, 2007a). Toll-like receptor-4 (TLR-4), a pattern recognition receptor that recognizes LPS, was also identified in both theca and granulosa layers, suggesting that it may mediate the changes in the expression of Av β D-12 by LPS stimulation (Subedi *et al.*, 2007b).

Although mRNA expression of Av β D-12 in the ovarian follicles has been identified, the localization of its protein

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molecule is still unknown. The effect of LPS on its localization is also waiting to be explained. Therefore, the objective of this study was to determine the localization of Av β D-12 protein in ovarian follicles and to examine the changes in their localization during follicular growth and in response to LPS. Antibody to Av β D-12 was raised using the synthetic peptide and used for immunocytochemical examination. Then, the following specific questions were addressed: (1) which follicular cells and tissue contain immunoreactive Av β D-12 (irAv β D-12), (2) whether their localization is changed with follicular growth, and (3) whether their localization is changed in response to LPS.

Material and Methods

Experimental Animals

White Leghorn hens (approximately 400-d-old) laying 5 or more eggs in a sequence were kept in individual cages under a 14 h light: 10 h dark regime. They were provided with free access to feed and water. Ovarian follicles were collected after euthanization of birds under anesthesia with sodium pentobarbital. Handling of the hens was conducted in accordance with the regulations of Hiroshima University for animal experiments.

Preparation of Rabbit Anti-Avian β -Defensin-12 Polyclonal Antibody

Synthetic peptide of the region of Av β D-12 amino acid sequence (Gly-Pro-Asp-Ser-Cys-Asn-His-Asp-Arg-Gly-Leu-Cys-Arg-Val-Gly-Asn-Cys-Asn-Pro-Gly-Glu-Tyr-Leu-Ala-Lys-Tyr-Cys-Phe-Glu-Pro-Val-Ile-Leu-Cys-Cys-Lys-Pro-Leu-Ser-Pro-Thr-Pro-Thr-Lys-Thr) reported by Xiao *et al.* (2004) was prepared by Scrum Inc. (Tokyo, Japan). They were conjugated to keyhole limpet haemocyanin (KLH; Wako Pure Chemical Industries, Osaka, Japan) (Kitagawa and Ailawa, 1976; Liu *et al.*, 1979). Two Japanese white rabbits were immunized by KLH conjugated Av β D-12 synthetic peptide (0.25 mg/rabbit) emulsified with an equal volume of Freud's complete adjuvant (Sigma-Aldrich Co., MO, USA). Serum was collected before and after immunization at two weeks interval to check the antibody titre by ELISA as described previously (Yoshimura *et al.*, 2007). The whole blood was collected on day 74 under anesthesia with sodium pentobarbital to prepare antiserum. Then, the anti-Av β D-12 antibody of this antiserum was purified using HiTrap affinity column conjugated with synthetic Av β D-12 peptide (GE Healthcare, Tokyo, Japan) according to the manufacturer's directions.

Tissue Collection

Healthy White Leghorn laying hens were i.v. injected with or without LPS at a dose of 1 mg/kg BW. This dose of LPS was sufficient to stimulate the mRNA expression of Av β D-12 (Subedi *et al.*, 2007a). The solution of LPS was prepared by dissolving LPS from *E. coli* 0111: B4 (Wako Pure Chemical Industries, Osaka, Japan) in Dulbecco's phosphate buffered saline (Nissui Pharmaceutical Co., Tokyo, Japan) at a concentration of 4 mg/mL.

White follicles (WF), the third largest follicles (F₃) and the largest follicles (F₁) were collected before and after 12 and 24 h of LPS injection ($n=3$ in each group). They were fixed in 10% formalin in PBS, dehydrated with graded ascending series of alcohols, cleared with xylene and embedded in paraffin. Then, sections (6 μ m) were prepared in MAS coated pre-cleaned slides (Matsunami Glass Ind., Japan) and air-dried.

Immunocytochemistry

Sections were deparaffinized with xylene and rehydrated with graded descending series of alcohols and washed with running water. Antigen retrieval was done in autoclave for 1 min by heating sections with antigen enhancing solution (0.1 M citric acid and 0.1 M trisodium citrate, pH 6.0). The immunocytochemistry was performed using Vecta Stain ABC kit (Vector Laboratories, Burlingame, CA, USA). After heating, the slides were allowed to cool and washed with PBS, followed by blocking with 1.5% (vol/vol) normal goat serum in PBS for 1 h at room temperature. Sections were incubated overnight with rabbit anti-Av β D-12 polyclonal antibody at a concentration 40 μ g/mL, followed by three times washing with PBS (3 $\times$ 5 min). The sections were then incubated with biotinylated anti-rabbit IgG and avidin-biotin-peroxidase complex for 1 h each and were washed three times with PBS (3 $\times$ 5 min) after each step. Immunoprecipitates were visualized by incubating the sections with 0.02% (wt/vol) 3', 3'-diaminobenzidine tetrahydrochloride and 0.005% (vol/vol) H₂O₂ in 0.05 M Tris-HCl buffer (pH 7.6). The sections were dehydrated with graded series of alcohols and mounted. They were examined under a light microscope (Nikon Eclipse E600, Tokyo, Japan) using a Normarsky filter.

Control staining was carried out simultaneously in which the first antibody was replaced with absorbed antibody or normal rabbit IgG. The absorbed antibody for Av β D-12 was prepared by incubating aliquots of antibody solutions overnight in rotator at 4°C with five times excess amount of corresponding peptide. Simultaneously, other sections were also stained with haematoxyline and eosin for the observation of the general tissue structure.

Results

Figure 1 shows the structures of WF and F₃. The wall of WF and F₃ consisted of perivitelline layer, granulosa layer, theca interna, theca externa and superficial layer (Fig. 1a and b). Granulosa layer consisted of 2–3 cell layers in WF, whereas did single cell layer in F₃. Theca interna was rich in cell components and the capillaries were developed in both WF and F₃. The theca externa was a dense connective tissue in both follicles. The structure of F₁ was same as F₃ (data not shown).

The distribution of irAv β D-12 in ovarian follicles is shown in Fig. 2. Immunoreaction products were not found in the theca and granulosa layers of WF (Fig. 2a), whereas they were found in the capillary-associated cells in the outer theca interna, granulosa cells and perivitelline

Fig. 1. Sections of hen ovarian follicles.

a) WF: White follicle. b) F₃: the third largest follicle. SL = superficial layer; TE = theca externa; TI = theca interna; G = granulosa layer; P = perivitelline layer (open arrows); O = oocyte; Y: yolk. Haematoxyline and eosin staining. Scale bars represent 40 μm.

layer of F₃ and F₁ (Fig. 2b and c). Immunoreaction products were not found in the sections of F₃ immunostained with absorbed antibody of AvβD-12 instead of first antibody (Fig. 2d). Staining was also not found when sections were immunostained with normal rabbit IgG (data not shown).

The irAvβD-12 was not found in the theca and granulosa layers of WF even after LPS injection (data not shown). In the F₃ follicles, the irAvβD-12 in the capillary-associated cells in outer theca interna (Fig. 3a) was decreased after 12 h (Fig. 3b) and disappeared after 24 h (Fig. 3c) of LPS injection, while it became negligible in the granulosa layers by 12 h of LPS injection (Fig. 3b). Perivitelline layer was kept positive for irAvβD-12 even after 24 h of LPS injection. The distribution pattern of irAvβD-12 was identified same in F₁ as in F₃ of LPS stimulated birds (data not shown). Changes in the localization of AvβD-12 in the yellow follicles (F₃ and F₁) in response to LPS injection were summarized in Table 1.

Discussion

This is the first report that identified irAvβD-12 protein in hen ovary and examined the changes in its localization with follicular growth and LPS stimulation. The noteworthy findings are as follows: 1) irAvβD-12 was localized in the theca interna, granulosa layer and perivitelline layer of yellow follicles but not found in those of WF, and 2) i.v. injection of birds with LPS resulted in the decrease of

Fig. 2. Sections of growing ovarian follicles immunostained for avian β-defensin-12.

a) WF: white follicle. b) F₃: the third largest follicle. c) F₁: the largest follicle. d) Control staining by absorbed antibody in F₃. Immunoreaction products are localized in the capillary-associated cells in the outer theca interna (long arrows), granulosa (short arrows) and perivitelline layer (open arrows). See Fig. 1 for other explanations. Scale bars represent 40 μm.

irAvβD-12 in the theca interna and granulosa layer.

IrAvβD-12 was found in the capillary-associated cells in the outer theca interna of yellow follicles. Capillary beds develop in the theca interna (Gilbert, 1979) and blood flow is higher in the larger follicles (Scanen *et al.*, 1982; Wolfenson *et al.*, 1982). Such increase of blood flow may facilitate the entry of pathogenic organisms to the theca interna, and may affect the synthesis of AvβD-12. In contrast, the theca externa may not be responsible to produce AvβD-12 because immunoreaction products were not found. The granulosa cells and perivitelline layer of yellow follicles were also positive for AvβD-12. Because many of the components of perivitelline layer are secreted by granulosa cells (Gilbert, 1979; Pan *et al.*, 2000; Sasanami *et al.*, 2004), AvβD-12 synthesized by granulosa cells may also be secreted to the perivitelline layer.

Fig. 3. Sections of the third largest follicles immuno-stained for avian β -defensin-12 at different time points after lipopolysaccharide stimulation. Follicles were collected before LPS injection (a), and after 12 h (b) and 24 h (c) of LPS injection. Immunoreaction products are localized in the capillary-associated cells in the outer theca interna (long arrows), granulosa (short arrow) and perivitelline layer (open arrows). See Fig. 1 for other explanations. Scale bars represent 40 μ m.

IrAv β D-12 in the granulosa and perivitelline layers may form a barrier preventing the bacterial invasion from the theca to yolk.

The theca and granulosa layers undergo a marked structural and functional differentiation during the process of follicular recruitment from prehierarchical to hierarchical phases. The theca layer of WF become distinguishable to theca interna with abundant capillaries and dense theca externa in hierarchical follicles (Gilbert, 1979), and the granulosa layer changes from 2 to 3 cell layers to a single cell layer (Fig. 1). Transport of yolk precursors from theca and granulosa layers to oocyte is limited to white yolk in WF, whereas it is possible for yellow yolk in yellow follicles (Gilbert, 1979; Johnson, 2000). Estrogen production in the theca was greater in WF than yellow follicles, and estrogen receptors were found in the granulosa of yellow follicles but not in that of WF (Bahr *et al.*, 1983; Yoshimura *et al.*, 1995). These structural and functional changes suggest that follicular cells differentiate during the follicular growth, and such cellular differentiation may be responsible in the increased synthesis of Av β D-12 protein in the theca and granulosa layers of yellow follicles. However, the synthesis of Av β D-12 in

Table 1. Summary of the results of immunocytochemistry for Av β D-12 in the theca, granulosa, and perivitelline layers of yellow follicles (F_3 and F_1) before and after LPS injection

Tissues	Time after LPS injection (h)		
	0	12	24
Theca externa	—	—	—
Theca interna	+ ¹⁾	+ ¹⁾ to —	—
Granulosa layer	+	—	—
Perivitelline layer	+	+	+

Note: + = positive for immunostaining.

— = negative for immunostaining.

¹⁾ = immunoreaction products appeared in the capillary-associated cells in the outer theca interna.

yellow follicles (F_3 and F_1) was similar. It is suggested that the distribution of Av β D-12 in F_3 and F_1 may not be affected by the follicular growth during this stage. The mRNA expression of Av β D-12 was constant in the theca and granulosa layers of both WF and yellow follicles (Subedi *et al.*, 2007a). A possibility is assumed for the discrepancy results between gene and protein expressions: although mRNA for Av β D-12 is expressed in WF, its protein synthesis may not be completed in the undifferentiated cells in the theca interna and granulosa cells. Thus, the Av β D-12 synthesizing ability may become developed in the theca interna and granulosa cells with the follicular growth from prehierarchical to hierarchical phases.

LPS injection resulted in the decrease of irAv β D-12 in both theca interna and granulosa layer of yellow follicles compared to non-injected follicles. The changes in the distribution pattern of irAv β D-12 in response to LPS injection were similar between F_3 and F_1 . In contrast, Av β D-12 mRNA was increased in the theca and decreased in the granulosa layer of yellow follicles in response to LPS (Subedi *et al.*, 2007a). TLR-4 that recognizes LPS was observed in the capillary-associated cells of the theca interna and granulosa cells of those follicles (Subedi *et al.*, 2007b). It is assumed that LPS recognized by TLR-4 in the capillary-associated cells of theca and granulosa cells leads to the changes in mRNA expression for the synthesis of Av β D-12 (Subedi *et al.*, 2007a, b). However, the synthesized protein might be released from the cells, resulting into the decrease in stored molecules within the cells. The decrease of irAv β D-12 in the granulosa cells in response to LPS might be the results from the decrease in the expression of its mRNA and/or from the release of the stored proteins from the cells. The secretion of Av β D-12 by the cells of theca and granulosa layers in response to LPS may play roles in the local defense against Gram-negative bacteria.

In conclusion, these results suggest that Av β D-12 protein is contained in the theca interna, granulosa and perivitelline layer of yellow follicles and its distribution is increased during follicular growth from a white follicle to a yellow follicle. The LPS stimulation is likely to cause the

decrease of this protein stored in the tissues, probably by inducing the secretion of them from the cells for the local host defense.

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