«Research Note»

Preparation and Application for Immunocytochemistry of Antibody to Gallinacin-3, an Antimicrobial Peptide, in Chicken

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The aim of this study was to develop an antibody to gallinacin-3 which is one of the antimicrobial peptides in chickens. The antibody was raised in rabbit using a synthetic peptide of gallinacin-3 (Gal-3), and purified through an affinity column. Specificity of antibody was examined by competitive ELISA using Gal-3-HRP and synthetic Gal-3, or the tissue peptides isolated from the uropygial gland and the uterus. Immunostaining was performed on sections of the trachea, uterus, vagina, cloacal gland and uropygial gland. In the competitive ELISA, co-incubation of Gal-3-HRP with Gal-3 and tissue peptides extracted from uropygialis and uterus caused a clear decline of optical density (A450) value in a dose dependent manner. The epithelial cells in the trachea, uterus, vagina, cloacal gland and uropygial gland stained positive using the prepared gallinacin-3 antibody. Sections incubated with antibody absorbed with Gal-3 (control staining) resulted in negative staining. These results suggest that the antibody to gallinacin-3 prepared in the current study is useful for immunocytochemical identification of gallinacin-3 in chickens. The current immunocytochemical results suggest the presence of gallinacin-3 in the epithelial cells of trachea, oviduct, cloacal gland and uropygialis.

Key words: antibody preparation, antimicrobial peptide, chicken, gallinacin

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Introduction

Defensins are small antimicrobial peptides that are produced by leukocytes and epithelial cells, and play an important role in innate immunity. Mammalian defensins are classified into three subfamilies, namely α -, β - and θ -defensins, which differ in their tissue distribution and the disulphide links between the six conserved cysteine residues (Andreu and Rivas, 1998; Klotman and Chang, 2006). The β -defensin family in poultry is termed gallinacins, which contains 3 intermolecular cysteine disulfide bonds (Harwig *et al.*, 1994). Currently, genes of 13 different gallinacins (gallinacin 1–13) have been reported, and their de-

duced peptides varied from 63 to 104 amino acid residues in length, all sharing the characteristic defensin motif (Lynn *et al.*, 2004; Xiao *et al.*, 2004). The mRNA expressions of gallinacin-1, -2, and -3 in cultured vaginal cells were increased upon stimulation with *Salmonella enteritidis* or lipopolysaccharide (LPS) within 24 h, suggesting that synthesis of these antimicrobial peptides in the vagina increases in response to these microbial components (Yoshimura *et al.*, 2006). Antimicrobial activity of synthetic peptide of gallinacin-11 has been demonstrated against a range of bacteria and was predominantly active against the intestinal pathogens *Salmonella typhimurium* and *Listeria monocytogenes* (Higgs *et al.*, 2005).

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Zhao *et al.* (2001) characterized gallinacin-3 gene structures and showed the epithelial expressions in several organs including trachea. The tracheal expression of gallinacin-3 increased significantly after experimental infection of chickens with *Haemophilus paragallinarum*, although its expression in the tongue, esophagus and bursa of Fabricius was unaffected. More recently, Xiao *et al.* (2004) reported that seven gallinacin genes (gallinacin-1 to -7) are predominantly expressed in bone marrow and the respiratory tract, whereas the other six genes (gallinacin-8 to -13) were more restricted to liver and urogenital tract. These reports strongly suggest that gallinacins play significant role in the innate immunity for the defense to microorganisms like the other antimicrobial peptides. However, the investigations for gallinacin expressions in the tissues were limited to the analysis of gene expressions until now. To identify the synthesized peptides in the tissues, the preparation of antibodies against gallinacin is essential. The aim of this study was to develop the antibody to gallinacin-3, the mRNA expression of which has been identified in the oviduct (Ohashi *et al.*, 2005), trachea and other organs containing epithelial tissues (Zhao *et al.*, 2001).

Materials and Methods

Birds and Tissue Collection

White Leghorn hens of approximately 400-d-old age and laying 5 or more eggs in a sequence were used. They were kept in individual cages under a 14 L:10D lighting regimen, and were provided with feed and water *ad libitum*. When the tissues were collected, the hens were euthanized under anesthesia with sodium pentobarbital at 18–20 h before estimated time of ovulation. Handling of chickens was done in accordance with the regulations of Hiroshima University for animal experiments.

Synthesis of Gallinacin-3 Peptide

A peptide (Gal-3; Arg-Phe-Pro-His-Ile-Ala-Ile-Gly-Lys-Cys-COOH) ranging from Arg 38 to Cys 47 of reported gallinacin-3 sequence existing in its matured region (Xiao *et al.*, 2004) was synthesized using Shimazu peptide synthesizer PSSM8 (Shimazu Co., Kyoto, Japan) as described previously (Iijima *et al.*, 2003). The synthesized peptide of Gal-3 was precipitated with cold diethyl ether and lyophilized. The peptide was then purified using Tosoh HPLC pump and UV-VIS detector (Tosoh, Tokyo, Japan), equipped with a Capcellpak C18 column (10×250

mm), eluted with a linear gradient of acetonitrile/0.1% trifluoroacetic acid. Aliquots of peptide purified by RP-HPLC was characterized with electrospray ionization/ion trap mass spectrometer (Thermo Fisher Scientific Inc., MA, USA) as described previously (Iijima *et al.*, 2003).

Preparation of Anti-Gallinacin-3 Antibody

The synthetic Gal-3 peptide (2 mg) was conjugated to 2 mg maleimide activated mariculture keyhole limpet hemocyanin (mcKLH; Piece Biotechnology Inc., IL, USA) according to the manufacturer's directions. The obtained conjugates (Gal-3-KLH) were dialyzed against 50 mM phosphate buffer, pH 7.4, containing 0.14 M NaCl. Three rabbits were immunized with Gal-3-KLH. The initial immunization was performed by subcutaneous injection with 0.15 mg Gal-3-KLH emulsified with Freund's complete adjuvant (Difco Lab., MI, USA), followed by the second immunization with 0.25, 0.3 or 0.35 mg antigen by a same method as the initial one on day 14. The final immunization was carried out on day 49 by intravenous injection with 0.3 mg Gal-3-KLH dissolved in saline. The whole blood was collected on day 56 under anesthesia with sodium pentobarbital, and then serum was prepared from each blood sample.

The titer of the antisera collected on days from 7 to 56 was determined by enzyme-linked immunosorbent assay (ELISA) using Gal-3 conjugated with bovine serum albumin (Gal-3-BSA) as antigen and peroxidase-labeled affinity-purified antibody to rabbit IgG (MP Biomedicals, OH, USA). The Gal-3-BSA conjugate was prepared by a similar procedure as the preparation of Gal-3-KLH described above using synthetic Gal-3 and mcBSA (Piece Biotechnology Inc.). Then, an affinity column with Gal-3-Toyopearl was prepared using Toyopearl AF-Epoxy-650M (Tosoh, Tokyo, Japan), and anti-Gal-3 antibody was purified with Gal-3-Toyopearl as described previously (Iijima *et al.*, 2003).

Specificity Analysis of Antibody by Competitive ELISA

A 96-well microplate was coated with 100 μ l of 2 μ g/ml Gal-3 antibody, followed by washing 4 times with PBS. After a treatment with blocking solution (0.5% BSA in PBS with 15% saccharose), the wells were incubated with 50 μ l of Gal-3-HRP diluted to 1:10,000 and 50 μ l of different concentrations of synthetic Gal-3 (0.0001 to 10 μ g/ml) or the tissue peptides isolated from uropygial gland and uterus (2.5

to 150 $\mu\text{g}/\text{ml}$) for 2 h. The wells were washed with PBS, incubated with 150 μl of Tetramethyl benzidine (TMB) solution for 30 min. The reaction was stopped by adding 50 μl of 4N H_2SO_4 , and optical density was measured at 450 nm wave length (A450).

For the above competitive ELISA, the Gal-3-HRP conjugate was prepared as described previously (Isobe *et al.*, 2005), using synthetic Gal-3 and horseradish peroxidase (Sigma Chem. Co., St Louis, MO, USA). The extraction of tissue peptide was performed by homogenizing in 20 mM HCl and 1 N CH_3COOH , followed by centrifugation at 2,200 g for 5 min. The supernatant was mixed with acetone (1:2, v/v) and centrifuged at 2,200 g for 20 min. A part of the supernatant was transferred into another tube, and acetone was removed at 70°C. Then, the sample was dissolved in 2 M Tris-HCl, pH 8.5, and filtrated through 0.22 mm filter. The concentrations of peptides were measured using protein assay reagent (Bio-Rad Lab, Hercules, CA) as described by the manufacturer.

Tissue Preparations and Immunostaining

The trachea, oviductal uterus and vagina, cloacal gland and uropygial gland were fixed with 10% formalin in PBS, and dehydrated with a graded series of alcohol and cleared in xylene, followed by embedding in paraffin as usual manner. Sections (4 μm thickness) were air-dried on slides. After deparaffinization, the sections were autoclaved in 2 mM citric acid (pH 6.9) for 1 min to activate antigen and washed with PBS. The sections were incubated with anti-Gal-3 antibody diluted at 1:100 in PBS with 0.5% BSA (PBS-BSA) for 4 h at room temperature. Then the sections were washed with PBS for 15 min (5 min $\times$ 3 times). The immunoreaction products were identified using VECTASTAIN ABC kit (Vector Lab. Inc., Burlingame, CA) according to manufacture's instruction. Briefly, sections were incubated with biotin-conjugated anti-rabbit IgG and avidin-biotin-peroxidase complex for 1 h each, followed by washing with PBS for 15 min (5 min $\times$ 3 times). Then, the immunoreaction products were visualized by incubating the sections with a reaction mixture consisting of 0.02% (w/v) 3, 3'-diaminobenzidine tetrahydrochloride and 0.005% (v/v) H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.6. The sections were counterstained with hematoxylin, dehydrated and covered. For the control staining, the first antibody was replaced with absorbed antibody that was

obtained by incubating 100 μg anti-gallinacin-3 antibody and 500 μg synthetic Gal-3 peptide overnight at 4°C. No specific staining was observed in the control staining.

Results

Figure 1 shows the results of competitive assay between the Gal-3-HRP and the Gal-3 peptide or tissue peptides extracted from uropygialis and uterus by ELISA. Co-incubation of Gal-3-HRP with Gal-3 resulted in a decrease of A450 value with an increasing amount of Gal-3 (Fig. 1a). The absorbance declined with co-incubation of Gal-3-HRP with tissue peptide from uropygialis and uterus, but not by the peptide of skeletal muscle and heart, in a dose dependent manner (Fig. 1b).

The uropygialis contained tubules with multilayered epithelium. Immunoreactivity of gallinacin-3 was observed in the cytoplasm of epithelial cells in the upper region of epithelium, but not in the basal region (Fig. 2a). The mucosal folds of the uterus consisted of the mucosal epithelium and lamina propria containing tubular glands. The tubular gland cells were not stained, whereas the tall and narrow cells in the mucosal epithelium were positive for gallinacin-3 immunostaining (Fig. 2b). The mucosal fold of the vagina was lined with ciliated pseudostratified epithelium, and the tall and narrow cells also stained positive for gallinacin-3 (Fig. 2c). The cloacal gland formed well developed primary and secondary folds lined with a single layer of epithelial cells. Some of the epithelial cells showed dense immunostaining for gallinacin-3, whereas the other cells were stained faintly (Fig. 2d). The mucosal tissue of the trachea consisted of ciliated pseudostratified epithelium and lamina propria. Gallinacin-3 immunoreactivity was identified in the cytoplasm of the mucosal epithelial cells. The lamina propria did not show any immunoreaction (Fig. 2e). Control staining was performed using absorbed antibody in place of the first antibody. No immunostaining was observed on the sections of uropygialis (Fig. 2f), uterus, cloacal gland and trachea (data not shown).

Discussion

We are reporting that an antibody to gallinacin-3 which was raised using synthetic peptide of 38–47 region of the amino acid sequence of primary gallinacin-3. Gallinacins are antimicrobial peptides

Fig. 1. Competitive assay between the synthetic gallinacin-3-HRP and the synthetic gallinacin-3 or tissue peptides extracted from tissues by ELISA. (a) Dose dependency of competition using 1 ng to 10 $\mu\text{g/ml}$ synthetic gallinacin-3 peptide. (b) Dose dependency of competition using 2.5 to 150 $\mu\text{g/ml}$ peptide isolated from uropygialis, uterus, skeletal muscle and heart.

that play essential role in the innate immunity for host defense. The nucleic acid sequences have been reported for 13 types of gallinacins, whereas antibodies of them were not yet available. Preparation of the antibodies for gallinacins is essential for the studies to identify the products of their gene expressions.

The competitive ELISA confirmed that the binding of the Gal-3-HRP to the gallinacin-3 antibody was significantly reduced by Gal-3 peptide. In addition, the peptides isolated from uropygialis and uterus, which showed positive immunostaining on sections, reduced the binding of Gal-3-HRP to the antibody in ELISA. These results suggest that the antibody recognized specifically Gal-3 that consisted of 10 peptides selected from the gallinacin-3 molecule, and also recognized gallinacin-3 molecules in the tissues.

When the antiserum without purification by affinity column was used for immunohistochemistry, no immunostaining was observed on any sections (data not shown). However, immunostaining using the antibody that was purified through affinity column with Gal-3-Toyopearl showed positive reaction in the uropygialis, uterus, vagina, cloacal gland and trachea, suggesting that the purification of antibody was necessary to use for immunocytochemistry. Furthermore, control immunostaining in these tissues using the antibody absorbed with Gal-3 peptide

showed negative staining. This result suggests that the antibody specifically recognizes gallinacin-3 in those tissues.

Strong mRNA expression of gallinacin-3 occurs in the trachea, bursa of Fabricius and tongue, whereas infection by *Haemophilus paragallinarum* enhanced the expression in the trachea (Zhao *et al.*, 2001). We also have reported that the expression of gallinacin-3 mRNA in the mucosal tissues of the uterus and vagina of laying hens was enhanced by inoculation with *Salmonella enteritidis* and LPS (Ohashi *et al.*, 2005; Yoshimura *et al.*, 2006). The current study localized immunoreactive gallinacin-3 in the mucosal epithelium of the trachea, uterus and vagina, suggesting that the expression of gallinacin-3 mRNA in these tissues resulted in the production of peptide molecules. It is suggested that the gallinacin-3 in these surface tissues of trachea and oviduct probably plays an important role in the defense against pathogenic microorganisms.

Although little is known whether mRNA expression of gallinacin-3 occurs in the cloacal gland and uropygialis, findings of immunoreaction products in the surface epithelium of these organs suggest that the epithelial cells produce gallinacin-3. The cloaca is the converging point for the digestive, urinary and reproductive tracts and hence is exposed to many microorganisms. The uropygialis secretes lipid-rich substances that may spread over the surface of the

Fig. 2. **Micrographs of tissue sections of laying hen immunostained for gallinacin-3.** a) Uropygialis. Upper epithelial cells (Ue) in the glandular tissue are stained immunopositive, whereas basal epithelial cells (Be) show negative staining. Dotted lines indicate the margin of each glandular structure. b) and c) Mucosal tissues of uterus and vagina, respectively. Immunopositive cells are located in the mucosal epithelium in both oviductal segments (arrows). d) Cloacal gland. Some of the epithelial cells lining the folds show dense staining (arrows), whereas others are moderately stained. e) Trachea. The ciliated epithelial cells show positive immunostaining (arrow). f) Negative control staining using absorbed antibody in the section of uropygialis. E=epithelium, LP=lamina propria. Scale bars=50 μ m.

body (Hodges, 1974). Gallinacin-3 synthesized in the epithelium of the cloacal gland may play a role in local defense against microorganisms. In the uropy-

gialis, gallinacin-3 produced by the epithelial cells may be incorporated into the secretory substances to add antimicrobial activity.

In conclusion, the anti-gallinacin-3 antibody prepared in the current study is useful for immunocytochemical identification of gallinacin-3. We could confirm the presence of the gallinacin-3 in the epithelial cells of trachea, oviduct, cloacal gland and uropygialis, partially supporting the previous reports on the expression of gallinacin-3 mRNA in various organs.

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