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Expression of tight junction molecule "claudins" in the lower oviductal segments and their changes with egg-laying phase and gonadal steroid stimulation in hens

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ABSTRACT

Tight junctions in the mucosal epithelium have essential roles as a mucosal barrier to prevent invasion of microbes into the hen oviduct tissue. The aim of this study was to determine the effects of the egg-laying phase and estradiol on the expression of tight junction molecule "claudins" in the lower oviductal segments in hens. White Leghorn laying and molting hens were used. Molting hens were given either sesame oil (vehicle) or estradiol benzoate (N = 5 per group) via injection. The lower segments of oviduct (isthmus, uterus, and vagina) of these birds were collected. Gene expression of claudin-1, -3, -5, lipopolysaccharide-induced *TNFα* factor (*LITAF*), and *IFNγ* was analyzed by quantitative reverse transcription polymerase chain reaction, and localization of claudin-1 was examined by immunohistochemistry. Permeability in the mucosal epithelium was assessed by intrauterine injection of fluorescein isothiocyanate-dextran. Expression of claudin-1, -3, and -5 genes and density of claudin-1 protein in the lower oviductal segments were higher in laying hens than in molting hens ($P < 0.01$); their expression was upregulated by estradiol ($P < 0.01$). Expression of *LITAF* and *IFNγ* genes was higher in molting hens than in laying hens. More fluorescein isothiocyanate-dextran infiltrated into the intercellular space of the uterus mucosal epithelium in molting hens than in laying hens and estradiol-treated molting hens. In conclusion, we inferred that barrier functions of the mucosal epithelium in the lower oviductal segments might be disrupted because of reduced claudin expression in molting hens, which might increase the susceptibility of mucosal tissue during the molting phase.

1. Introduction

In hens, microorganisms colonizing the cloaca might ascend the oviduct through the vagina and uterus. Mucosal barrier systems formed by epithelial cell junctions, mucin layer, and leukocyte activity, all have primary roles to prevent infection. Tight junctions (TJ) of mucosal epithelium are responsible for a paracellular barrier that protects underlying tissue against most toxic materials or pathogens [1–3]. Members of the claudin protein family form the main components of TJ; each family member laterally interacts with another claudin protein located in the adjacent cell in a homotypic or heterotypic manner [4,5]. They assemble in the plasma membrane, together with other proteins of the TJ complex, e.g., occludin and tricellulin [2,6]. Currently, nucleotide sequences of 3 claudins (claudin-1, -3, and -5) have been reported and 15 claudins (claudin-2, -4, -8, -10 to -12, -14 to -20, and -22 to -23) are predicted in the database of the National Center of Biotechnology Information [7]. Claudin-1 was present in high-resistance epithelia and crucial for the epidermal barrier. Claudin-3 was present in the tighter segment of the nephron, whereas claudin-5 constituted TJ strands in endothelial cells [8,9]. Thus, claudin-1, -3, and -5 are likely to be expressed to form TJs in

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epithelial tissues. In birds, the level of claudin-1 mRNA in immature testes was higher than that of adult testes of pheasant (*Phasianus colchicus*) [10]. Furthermore, expression of claudin-3, -5, and -16 in the intestinal epithelium was higher in 2-day-old chicks than in 20-day-old embryos [11]. Thus, claudin synthesis might be affected by physiologic and environmental factors.

Previous reports suggested that the oviduct was more susceptible in molting hens than laying hens because contamination of eggs by *Salmonella* was more frequent after resumption of laying in postmolting hens [12]. If expression of claudins in the oviductal epithelium declines, mucosal barrier functions might be weakened, leading to increased susceptibility to pathogenic agents. Proinflammatory cytokines such as *TNFα* and *IFNɣ* downregulated expression of claudins and increased paracellular permeability in the epithelium [13–15]. We reported a reduction of mucin expression in the oviduct during the molting phase, which might reduce the mucin barrier on the tissue surface [16]. However, it remains unknown whether expression of claudin in the oviduct changes during the molting phase, compared with that in the laying phase. It is also unknown whether expression of TJ-related molecules is regulated by estrogen, although the growth of the oviduct is upregulated by this steroid [17].

The goals of this study were to determine differences in expression of claudins in the lower oviductal segments (vagina, uterus, and isthmus) during various egg-laying phases, namely, laying and molting, and the role of estradiol in their expression in hens. Specific objectives were to determine whether: (1) expression of claudin-1, -3, and -5, (2) expression of lipopolysaccharide-induced *TNFα* factor (*LITAF*) and *IFNɣ*, and (3) permeability of fluorescein isothiocyanate (FITC)-dextran within mucosal epithelium in the lower oviductal segments, differed between laying and molting phases and if they were affected by estrogen stimulation.

2. Materials and methods

2.1. Experimental birds

Healthy White Leghorn laying and molting hens of approximately 500 days old were kept in individual cages under a daily light regimen of 14 hours light:10 hours dark. Hens regularly laying four or more eggs in a clutch were provided with feed and water *ad libitum*. Molting hens were given restricted feed (25 g/d) and free access to water, which induced cessation of egg-laying after 5 to 7 days. They were used after 20 days of cessation of egg-laying as the molting hen group. A proportion of the molting hens were given 1 mg β-estradiol benzoate (Sigma-Aldrich Co., St. Louis, MO, USA; EB group) or 100 μL of sesame oil (control group) im once daily for 7 days. The birds were handled in accordance with regulations of Hiroshima University for animal experiments.

2.2. Analysis of claudin and cytokine expression

Anesthesia was induced with Somnopentyl (Kyoritsu Seiyaku Inc., Tokyo, Japan), birds were euthanized, and the isthmus, uterus, and vagina of the laying hens (6 hours after oviposition), molting hens, estrogen-treated molting hens, and control hens (N = 5 per group) were collected.

2.2.1. Quantitative RT-PCR analysis for expression of claudins, LITAF, and IFNɣ

Quantitative reverse transcription (RT) polymerase chain reaction (PCR) analysis was performed as described [16]. Briefly, total RNA was extracted from the mucosal tissues of the isthmus, uterus, and vagina of each bird using Sepasol RNA I Super (Nacalai Tesque Inc., Kyoto, Japan). Extracted total RNA samples were dissolved in TE buffer (10 mM Tris, pH 8.0, with 1 mM EDTA), treated with 1 U of RQ1 RNase-free DNase (Promega Co., Madison, WI, USA), and put in a PTC-100 programmable thermal controller (MJ Research Inc., Waltham, MA, USA), programmed at 37 °C for 45 minutes and 65 °C for 10 minutes. Concentration of RNA in each sample was measured using Gene Quant Pro (Amersham Pharmacia Biotech, Cambridge, UK).

The RNA samples were reverse-transcribed using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan), according to the manufacturer's instructions. The reaction mixture (10 μL) consisted of 1 μg of total RNA, 1× RT buffer, 1 mM dNTP mixture, 20 U RNase inhibitor, 0.5 μg of oligo(dT)20 primer, and 50 U ReverTra Ace. Reverse transcription was performed at 42 °C for 30 minutes, followed by heat inactivation for 5 minutes at 99 °C using the PTC-100 Programmable Thermal Controller (MJ Research Inc.).

Primers for claudins, *LITAF*, *IFNɣ*, and *RPS17* PCR are shown (Table 1), with PCR performed using Takara Ex Taq (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. The PCR mixture (25 μL) contained 0.5 μL of cDNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1.25 U Takara Ex Taq, and 0.5 μM each primer. Amplification was performed in a PTC-100 Programmable Thermal Controller (MJ Research Inc.) under the following conditions: 94 °C for 30 seconds, and then 33 cycles at 95 °C for 30 seconds for denaturing, 65 °C for 30 seconds for annealing, and 72 °C for 45 seconds for extension. Thereafter, PCR products were separated by electrophoresis on a 2% (wt/vol) agarose gel containing 0.4% (wt/vol) ethidium bromide.

Real-time PCR was performed using a Roche Light Cycler system (Roche Applied Science, Indianapolis, IN, USA). The

Table 1
Primer sequences for claudins, *LITAF*, *IFNɣ*, and *RPS17*.

Target genes	Sequences 5′–3′	Accession number [reference]
Claudin-1	F: GAC TCG CTG CTT AAG CTG GA R: AAA TCT GGT GTT AAC GGG TG	AY750897 [10]
Claudin-3	F: AGC CCT CCA TCT CAG CAG R: TTC TCC GCC AGA CTC TCC	NM_204202 [11]
Claudin-5	F: GTC CCG CTC TGC TGG TTC R: CCC TAT CTC CCG CTT CTG G	NM_204201 [11]
LITAF	F: TGT GTA TGT GCA GCA ACC CGT AGT R: GGC ATT GCA ATT TGG ACA GAA GT	AY765397 [18]
IFNɣ	F: ACT GAG CCA GAT TGT TTC GAT GT R: TGC CAT TAG CAA TTG CAT CTC CT	X99774 [19]
RPS17	F: AAG CTG CAG GAG GAG GAG AGG R: GGT TGG ACA GGC TGC CGA AGT	NM_204217 [16]

Abbreviations: F, forward; R, reverse.