Differences in the mucosal surface barrier formed by mucin in the lower oviductal segments between laying and molting hens

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ABSTRACT The aim of this study was to determine the differences in the mucin expression that forms a mucosal surface barrier in the oviduct between laying and molting hens. The lower segments of oviducts (isthmus, uterus, and vagina) of White Leghorn laying and molting hens were collected. Localization and gene expression of mucosal mucin were analyzed by quantitative reverse-transcription PCR of mucin mRNA and mucin5AC immunohistochemistry. Sugar residues were localized by lectin (WGA or Jacalin) histochemistry. Expression of mucin mRNA was significantly declined in the lower oviductal segments in molting compared with laying hens. Immunoreactive-mucin5AC was localized in the mucosal epithelium and on the epithelial surface of laying hens, whereas it was reduced in molting hens. Substances positively stained by WGA and Jacalin were identified on the surface of the mucosal epithelium in the lower oviductal segments in laying and molting hens. These results suggest that mucin synthesis in the lower segments of the oviduct is reduced, although the existence of WGA- and Jacalin-positive sugars may be kept even in the molting phase. The reduction of mucin synthesis may result in a decline of mucosal barrier function in the molting phase.

Key words: chicken oviduct, molting, lectin, mucin glycoprotein

2012 Poultry Science 91:1173–1178
http://dx.doi.org/10.3382/ps.2011-02079

INTRODUCTION

The mucosal tissue of the oviduct fulfills defense functions that are essential for maintaining its health. Mucosal epithelial cells form a contagious lining that acts as a barrier against the moist exterior environment. The surface of the epithelial cell lining is covered by a mucus layer that protects the underlying epithelium from pathogenic microorganisms (Corfield et al., 2000; Perez-Vilar, 2007; Linden et al., 2008b). Mucins are composed of glycoproteins and secreted by mucosal epithelium (Gendler and Spicer, 1995; Linden et al., 2008b). They can be divided into 3 distinct subfamilies: 1) secreted gel-forming mucins, 2) cell-surface mucins, and 3) secreted non-gel-forming mucins. Gel-forming mucins, such as mucin5AC, are the major constituents of mucus and confer its viscoelastic properties, and cell-surface mucins are a prominent feature of the apical glycocalyx of the mucosal epithelia (Linden et al., 2008b). Sugar residues may be incorporated into the glycoprotein of mucin and also be responsible for the attachment of parasites and microbes to the mucosal epithelium (Van Poucke et al., 2010).

Mucin5AC, a secreted gel-forming mucin, was reported to be expressed in various tissues, including the respiratory tract, stomach, and cervix, in mammals (Porchet et al., 1995; Gipson et al., 1997; Buisine et al., 1998; Bara et al., 2003). Previous studies in chicks showed increased mucin mRNA expression in the intestine by starvation (Smirnov et al., 2004), probiotic feeding (Smirnov et al., 2005), and in ovo administration of carbohydrates (Smirnov et al., 2006). Immunoreactive mucins, including mucin5AC, in the distal colonic epithelium of mice were reported to be decreased by infection with *Citrobacter rodentium* (Lindén et al., 2008a). Thus, mucin synthesis may be affected by physiological and environmental factors in mucosal tissues.

The lower segment of the oviduct is the primary tissue where microorganisms colonizing in the cloaca ascend the oviduct. The hen oviduct may be more susceptible to pathogens during the molting than laying phase because contamination of eggs by *Salmonella* organisms was identified more frequently just after resumption of egg laying after molting (Keller et al., 1995; Holt, 2003). T-cell-mediated immune functions may be declined in the oviductal mucosa in molting hens because its frequency on the mucosal surface layer was reduced (Yoshimura et al., 1997). However, it remains unknown whether the mucosal surface barrier formed by mucin in the lower oviductal segments is changed in molting hens. Thus, the goal of this study was to determine differences in the mucin expression that forms the muco-

Received December 5, 2011.
Accepted January 29, 2012.
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sal surface barrier in the lower oviductal segments (vagina, uterus, and isthmus) between laying and molting hens. The specific questions were whether there are any differences in the protein localization and gene expression of mucin and in the localization of WGA lectin- and Jacalin lectin-positive sugar substances that may be incorporated into mucin in the lower oviductal segments between laying and molting hens. Lectins bind to a specific sugar residue of glycoprotein with high affinity. The WGA lectin, which is a lectin from wheat germ agglutinin (*Triticum vulgaris*), binds specifically to *N*-acetylglucosamine (**GlcNAc**) and *N*-acetylneuraminic acid (sialic acid). Jacalin lectin, the major protein from jackfruit (*Artocarpus heterophylus*) seeds, shows highly specific binding to galactose (**Gal**) and *N*-acetylgalactosamine (**GalNAc**; Kabir, 1998; Tatsuzuki et al., 2009; Fallis et al., 2010). Jung et al. (2011) reported that the epithelium and tubular gland cells of hen oviductal magnum were positive for WGA lectin, suggesting that these cells contained GlcNAc and sialic acid residues.

MATERIALS AND METHODS

Experimental Birds

Healthy White Leghorn laying and molting hens (approximately 500 d old; n = 5 each) were kept in individual cages under a daily light regimen of 14L:10D. The laying hens were provided with feed and water ad libitum and regularly lay 4 or more eggs in a clutch. They were used 6 h after oviposition. Molting hens were given a restricted feed (25 g/d) and free access to water, which induced cessation of egg laying after 5 to 7 d of treatment. They were used 20 d after cessation of egg laying as the molting hen group. The isthmus, uterus, and vagina were collected after euthanasia under anesthesia with Somnopentyl (Kyoritsu Seiyaku Inc., Tokyo, Japan). They were processed into paraffin sections and for total RNA sample preparation. The birds were handled in accordance with the regulations of Hiroshima University for animal experiments.

Quantitative Reverse-Transcription PCR Analysis for Expression of Mucin

Quantitative reverse-transcription PCR analysis was performed as described previously (Nii et al., 2011). Briefly, total RNA was extracted from the mucosal tissues of the isthmus, uterus, and vagina using Sepasol RNA I Super (Nacalai Tesque Inc., Kyoto, Japan). The extracted total RNA samples were dissolved in TE buffer [10 m*M* Tris (pH 8.0) with 1 m*M* EDTA]. They were treated with 1 U of RQ1 RNase-free DNase (Promega Co., Madison, WI) on a PTC-100 programmable thermal controller (MJ Research Inc., Waltham, MA), programmed at 37°C for 45 min and 65°C for 10 min. The 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 the total RNA, 1× RT buffer, 1 m*M* dNTP mixture, 20 U of RNase inhibitor, 0.5 μg of oligo(dT)20 primer, and 50 U of ReverTra Ace. Reverse transcription was performed at 42°C for 30 min, followed by heat inactivation for 5 min at 99°C using the PTC-100 Programmable Thermal Controller (MJ Research Inc.).

The PCR was performed using Takara *Taq* (Takara Bio Inc., Shiga, Japan), according to the manufacturer`s protocol. Primers used in this study are shown in Table 1. The PCR mixture (25 μL) contained 0.5 μL of cDNA, 1× PCR buffer, 1.5 m*M* $MgCl_2$, 0.2 m*M* each dNTP, 1.25 U Takara *Taq*, and 0.5 μ*M* of each primer. Mucin was amplified in a PTC-100 Programmable Thermal Controller (MJ Research Inc.) under the following conditions: 94°C for 30 s, then 34 cycles at 95°C for 30 s to denature, 58°C for 60 s to anneal, 72°C for 60 s for extension. The 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 the Roche Light Cycler system (Roche Applied Science, Indianapolis, IN). The reaction mixture (20 μL) contained 3 μL of cDNA, 1× SYBR Premix EX *Taq* (Takara), and 0.5 μ*M* of each primer. The mixture was placed in 20-μL capillaries (Roche Diagnostics GmbH, Mannheim, Germany). The cycle parameters for the PCR reaction were 95°C for 5 s and 62°C for 20 s. Data analysis was performed as described by Nii et al. (2011). Briefly, the Δ threshold cycle ($\mathbf{C_T}$) was calculated for each sample by subtracting the C_T value of RPS17 (internal control) from the C_T of the respective target gene. For relative quantification, the ΔC_T value of RPS17 was then subtracted from the ΔC_T of each experimental sample to generate the $\Delta\Delta C_T$. The $\Delta\Delta C_T$ value was therefore fit to the formula $2^{-\Delta\Delta CT}$ to calculate the approximate fold difference. The results are expressed as

Table 1. Primer sequences for mucin and RPS-17

Target gene	Sequence 5′–3′	Accession number, reference
Mucin	F: TCT TCC GCT ACC CTG GGC TCT GTAA R: CTC ATG CAG TTC TAG CAA GAT ACT	AJ487010, Smirnov et al., 2004
RPS-17	F: AAG CTG CAG GAG GAG GAG AGG R: GGT TGG ACA GGC TGC CGA AGT	NM_204217, Nii et al., 2011

fold change obtained from the ratio of the experimental samples and standard sample.

Mucin Immunohistochemistry

Tissue samples of the isthmus, uterus, and vagina were fixed in 10% (vol/vol) formalin-PBS, dehydrated, and embedded in paraffin. Sections (4-μm thickness) were air-dried on slides. The deparaffinized sections were autoclaved in 10 m*M* sodium citrate, pH 6.0, for 1 min. After washing in PBS (3 times for 5 min each), the sections were incubated with 5% (vol/vol) normal goat serum for 30 min, followed by overnight incubation with the mouse anti-mucin5AC monoclonal antibody (Santa Cruz Biotech. Inc., Santa Cruz, CA) diluted at a concentration of 4 μg/mL in PBS. After washing in PBS (3 times for 5 min each), the tissue sections were incubated with biotinylated anti-mouse IgG (Vector Lab. Inc., Burlingame, CA) for 1 h. After washing in PBS (3 times for 5 min each), sections were incubated with avidin-biotin-peroxidase complex (Vector Lab. Inc.) for 1 h. After washing in PBS (3 times for 5 min each), the immunoreaction products were visualized using 0.02% (wt/vol) 3′,3′-diaminobenzidine-tetrahydrochloride and 0.005% (vol/vol) H_2O_2 in 0.05 *M* Tris-HCl, pH 7.6 (DAB-H_2O_2 mixture), and counterstained with hematoxylin. Some sections were stained by hematoxylin and eosin for general histology. All sections after immunostaining and hematoxylin and eosin staining were dehydrated with graded series of alcohols and covered.

Lectin Histochemistry

To examine the localization of sugar residues in the mucosal barrier, the paraffin sections were stained by WGA or Jacalin lectins, as described previously (Utsumi and Yoshimura, 2011) with minor modifications. Briefly, the sections were deparaffinized and incubated with 5% (vol/vol) normal goat serum for 30 min to block nonspecific staining. The tissue sections were incubated overnight with biotinylated WGA (J-Oil Mills Inc., Tokyo, Japan) or biotinylated Jacalin (Vector Lab. Inc.) diluted at 20 μg/mL in PBS. After washing in PBS (3 times for 5 min each), sections were incubated with avidin-biotin-peroxidase complex (Vector Lab. Inc.) for 1 h. After washing in PBS (3 times for 5 min each), lectin binding was visualized using a DAB-H_2O_2 mixture. Slides were counterstained with hematoxylin, dehydrated with graded series of alcohols, and covered.

Statistical Analysis

Fold changes in the mucin expressions were expressed as the mean ± SEM, and the significance of differences between laying and molting groups was examined by *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Histological observation showed that the surface of the mucosal epithelium in each oviductal segment was lined by ciliated pseudostratified epithelium, and tubular glands were developed in the lamina propria of the isthmus and uterus of laying hens. In molting hens, the height of the mucosal epithelium was decreased in each segment and the tubular glands in the isthmus and uterus were regressed (data not shown).

Figure 1 shows differences in the mucin mRNA expression in the vagina, uterus, and isthmus between laying and molting hens. The expression was identified in all segments in laying hens; however, it was reduced markedly in molting hens with significant differences between laying and molting groups within each segment.

Figure 2 shows the localization of immunoreactive mucin5AC in the vagina, uterus, and isthmus. In laying hens, the immunoreaction products were identified on the surface of the mucosal epithelium of each segment (Figure 2a-c). Epithelial cells in each segment contained dense immunoreaction products in their cytoplasm (Figure 2a-c). Tubular gland cells in the uterus also contained immunoreaction products (Figure 2b). In contrast, in molting hens, immunoreaction products on the surface of mucosal epithelium were negligible in the vagina, uterus, and isthmus (Figure 2d-f). Immunoreaction products were observed in the cytoplasm of mucosal epithelial cells of the vagina (Figure 2d), whereas it was negligible in those of the uterus and isthmus (Figure 2e,f).

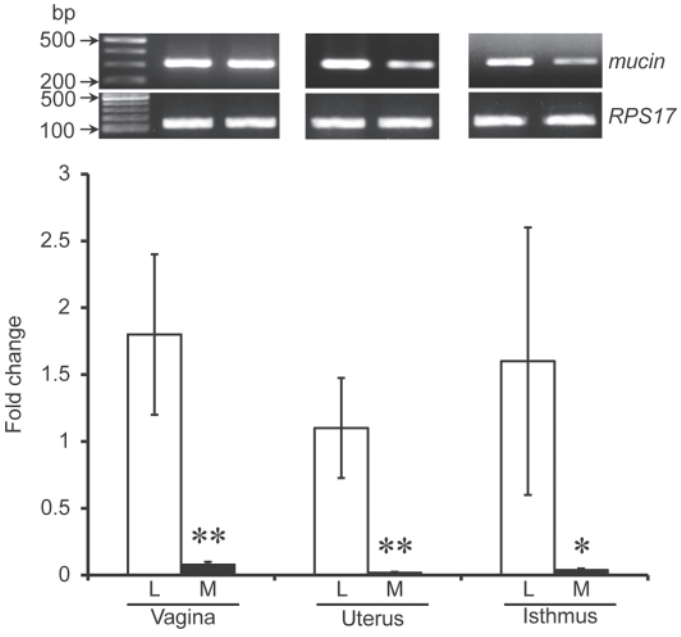

Figure 1. Differences in mucin mRNA expression in the vagina, uterus, and isthmus between laying and molting hens. Values are the mean ± SEM of fold change (n = 5). Asterisks indicate that values are significantly different between laying and molting hens (*$P < 0.05$, **$P < 0.01$). L = laying group, M = molting group.

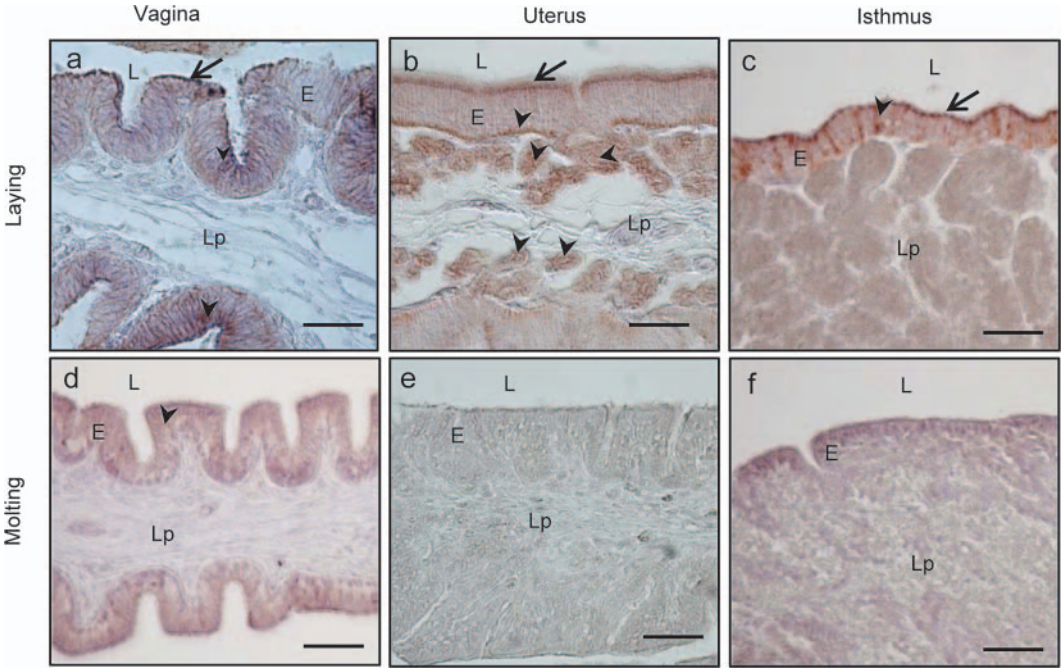

Figure 2. Localization of immunoreactive mucin5AC in the vagina, uterus, and isthmus of laying and molting hens (a–c = laying goup, d–f = molting group; a and d = vagina, b and e = uterus, c and f = isthmus). In laying hens, immunoreaction products are identified on the surface of mucosal epithelium of each segment (arrows). Epithelial cells of each segment and tubular gland cells in the uterus contain immunoreaction products in their cytoplasm (arrowheads). In molting hens, immunoreaction products on the surface of mucosal epithelium are negligible in each segment. E = mucosal epithelium, L = lumen of oviduct, Lp = lamina propria. Scale bars = 50 μm. Color version available in the online version of this paper.

The results of WGA- and Jacalin-lectin histochemistry are shown in Figure 3. In laying hens, WGA-positive and Jacalin-positive substances were localized in association with cilia on the surface of mucosal epithelium of the vagina, uterus, and isthmus (Figure 3a-f). These positive substances were not observed at the pits of the secondary mucosal folds (Figure 3a-f). Substances positive for each WGA and Jacalin were also identified in molting hens (Figure 3g-l) as observed in laying hens (Figure 3a-f).

DISCUSSION

We, here, report differences in the synthesis of mucin that may play a role in the mucosal barrier of the oviduct between laying and molting hens. Significant findings were 1) expression of mucin mRNA was higher and immunoreactive mucin5AC on the surface of mucosal epithelium was denser in laying than molting hens and 2) WGA- and Jacalin-positive substances were identified on the mucosal surface in both laying and molting hens.

The current study showed expression of mucin mRNA in the vagina, uterus, and isthmus, and localized immunoreactive mucin5AC on the apical surface and in the cytoplasm of mucosal epithelium of these oviductal segments in laying hens. It is possible that the identified mucin5AC could be related to the expression of the mucin gene examined in this study. A fragment of chicken mucin cDNA prepared using the same primers as this study had 67% homology to human mucin5AC (Smirnov et al., 2004). The mucin mRNA and immunoreactive mucin5AC were markedly reduced or negligible in the mucosa in molting hens. These results suggest that mucin synthesis was reduced in the 3 lower oviductal segments during molting.

Mucin5AC, a gel-forming mucin, is the major constituent of mucus and confers its viscoelastic properties (Desseyn et al., 2000). It may prevent pathogen penetrance by inhibiting bacterial adhesion to the mucosal epithelium surface (Berry et al., 2002). Mucins are composed of a peptide core containing heavily glycosylated regions and form a surface network above the epithelia (Carlstedt et al., 1983; Shambaugh et al., 1988). They have direct and indirect roles in defense from infection; namely, they have the ability to form a physical barrier and act as adhesion decoys (Linden et al., 2008b). The ability of the mucus layer to protect the epithelium from different pathogens is often attributed to the presence of charged groups in mucin molecules (Shambaugh et al., 1988). Not only do microbes bind to the sugar residue of mucin, but also, in some cases, mucins either have direct antimicrobial activity or carry other antimicrobial molecules (Lindén et al., 2008a). Cell-surface mucins may also initiate intracellular signaling in response to bacteria, suggesting that they have both a barrier and reporting function on the apical surface of mucosal epithelial cells (Linden et al., 2008b). The current study indicated that gene and protein expressions of mucin were significantly decreased in the lower segments of the oviduct in molting hens. These results suggest that the barrier function on the

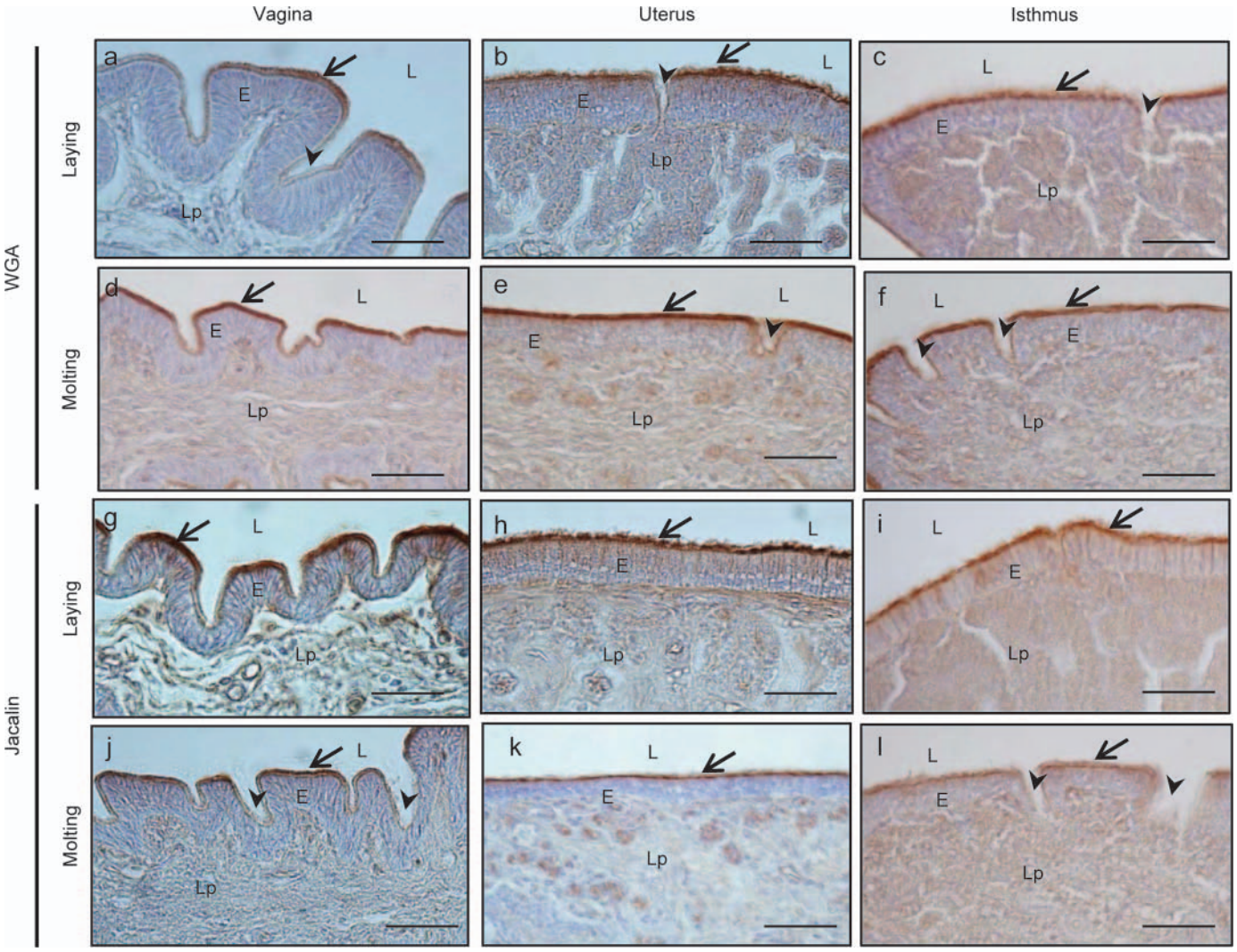

Figure 3. Lectin histochemistry using WGA (wheat germ agglutinin) and Jacalin in the vagina, uterus, and isthmus of laying (a–c, g–i) and molting hens (d–f, j–l). a–f = WGA staining; g–l = Jacalin staining; a, d, g, j = vagina; b, e, h, k = uterus; c, f, i, l = isthmus. Both WGA-positive and Jacalin-positive substances are localized in association with cilia on the surface of mucosal epithelium of each segment of laying and molting hens (arrows). Positive substances are not observed in the pits of the secondary mucosal folds (arrowheads). E = mucosal epithelium, L = lumen of oviduct, Lp = lamina propria. Scale bars = 50 μm. Color version available in the online version of this paper.

mucosal surface by mucin may be declined in the oviductal segments of molting hens.

The WGA- and Jacalin-lectin positive products were identified in association with the cilia on the surface of mucosal epithelium in both laying and molting hens. These results suggest that glycosylation by sugars recognized by these lectins occurs in substances on the surface of the mucosal epithelium, including mucin glycoprotein or the cell membrane. The glycocalyx of the mucosal surface may act as an effective barrier against invasion by pathogenic microorganisms and injury from toxic substances (Buckley et al., 2000). Lectin histochemistry did not show differences in the localization of WGA- and Jacalin-positive substances in the vagina, uterus, and isthmus between laying and molting hens. These sugar substances may be incorporated not only into mucin but also into other mucosal surface components and maintained even in the molting phase.

In conclusion, the current results suggest that mucin synthesis in the lower segments of the oviduct is reduced, although the localization profile of the WGA- and Jacalin-positive sugar residue may not be changed in molting hens. The reduction of mucin synthesis in the molting phase may result in the decline of mucosal barrier functions, leading to greater susceptibility to pathogens than in the laying phase.

ACKNOWLEDGMENTS

This work was supported by Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

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