Immunolocalization of avian β -defensins in the hen oviduct and their changes in the uterus during eggshell formation

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Abstract

The aim of this study was to examine whether avian β -defensin proteins (av β Ds) exist in the oviduct, and whether those in the uterus are secreted to the eggshell membrane and eggshell. The oviducts of White Leghorn hens at different times of egg formation, eggshell membrane, and eggshell were used. The presence of immunoreactive (ir) av β D-3, -11, and -12 was examined by immunohistochemistry and western blot. Two or three types of av β Ds were identified in the mucosal surface epithelial cells in each oviductal segment.

The density of ir-av β D-3 and -12 in the uterus was decreased after the egg entered this segment. Western blot analysis confirmed the presence of ir-av β D-3, -11, and -12 in the uterus. In the eggshell membrane, only ir-av β D-3 was detected on the surface of fibers at the outer layer of the membrane. The ir-av β D-3, -11, and -12 were identified in the eggshell matrix by western blot. These results suggest that the surface epithelial cells are the major sites where av β Ds proteins exist, and the av β Ds secreted by the uterus cells are likely to be incorporated in the eggshell membrane and eggshell. These av β Ds may play roles in the innate host defense of the oviduct and egg surface.

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Introduction

The hen oviduct consists of five anatomically and functionally distinct segments, namely the infundibulum, magnum, isthmus, uterus, and vagina (Palmer & Guillette 1988). During the passage of the egg through the oviduct, the clava is secreted on the surface of the ovum in the infundibulum, whereas albumen, eggshell membrane, and eggshell components are secreted in the magnum, isthmus, and uterus respectively. The completely developed egg is oviposited through the vagina and cloaca (Gilbert 1979).

The hen oviduct may be infected by various pathogenic microorganisms such as *Salmonella enteritidis* (Barnhart *et al.* 1993) and *Mycoplasma meleagridis* (Yamamoto & Herrad 1966), which may cause the functional disorder of the oviduct and contamination of eggs. The immune function of the oviduct is essential to protect the oviductal tissues from infection as well as for production of hygienic eggs.

Defensins are very common cationic antimicrobial peptides among vertebrates, which play significant roles in innate immunity, and are characterized by the presence of conserved cysteine-rich defensin motifs. Based on the spacing pattern of cysteines, they are divided into α , β , and θ subgroups (Haryadi & Pak 2004, Xiao *et al.* 2004). The defensins in birds belong to β -defensins group (avian β -defensins: av β Ds) and are active against Gram-positive and Gram-negative

bacteria, protozoans, as well as some fungi and enveloped viruses (Sugiarto & Yu 2004, Xiao *et al.* 2004, van Dijk *et al.* 2008). In addition, they may have a chemotactic function because they can attract T-cells, monocytes, and immature dendritic cells while having toxic effects on a wide range of normal and malignant cells including those resistant to tumor necrosis factor in mammals (Bulet *et al.* 2004).

Fourteen types of av β D genes have been identified, and at least 11 of these are expressed in the hen oviduct (Xiao *et al.* 2004, Yoshimura *et al.* 2006, Abdel Mageed *et al.* 2008). Ohashi *et al.* (2005) showed the mRNA expression of av β D-1, -2, and -3 in the surface epithelium of the vagina using *in situ* hybridization. However, the presence of av β D proteins in the hen oviduct remains unknown.

If the av β D proteins are synthesized, they may play roles in the local host defense in the oviduct. Furthermore, if they are secreted to the egg contents during the egg formation, they may participate in the defense of eggs against microorganisms. One of the most important structures of eggs for defense against foreign agents is the eggshell and eggshell membrane that are reported to contain antimicrobial substances (Ahlborn *et al.* 2006, Wellman-Labadie *et al.* 2008). It is hypothesized that those antimicrobial substances are synthesized in the uterus and secreted to be incorporated in the eggshell membrane and eggshell during shell formation. Thus, the aim of this study was to determine

the localization of these av β Ds in the oviduct of laying hens, and the possibility that av β D proteins synthesized in the uterus are secreted to the eggshell membrane as well as eggshell. The specific questions were: 1) which cells in the oviduct contain av β D proteins, 2) whether the density of av β D proteins in the uterus is changed during shell formation, and 3) whether eggshell membrane and eggshell contain av β D proteins.

We have reported that the expression of five types of av β Ds was significantly increased in response to i.v. injection with lipopolysaccharide (LPS), suggesting that synthesis of these av β Ds responds to bacterial components (Abdel Mageed *et al.* 2008). Among them, we selected av β D-3, -11, and -12 to identify the molecules because they were the three most sensitive av β Ds that responded to LPS.

Results

Immunolocalization of av β D-3, -11, and -12 in the oviduct

Immunoreaction products of av β D-3 were found in the supranuclear cytoplasm of basal cells of the surface epithelium in the cephalic region of the magnum, isthmus, uterus, and vagina, whereas the density of the positive cells was highest in the uterus (Fig. 1B and D–F). On the other hand, the basal cells of the caudal region of the magnum showed a weak staining (Fig. 1C). The immunoreaction products were also found in the tubular gland cells in the magnum (cephalic and caudal regions) and isthmus (Fig. 1B–D). No cells of the infundibulum showed immunolabeling for av β D-3 (Fig. 1A).

Figure 2 shows the localization of the immunoreaction products for av β D-11. Fine granules of immunoreaction products were identified in the basal cells of the surface epithelium of the infundibulum (Fig. 2A). In the cephalic region of magnum, only the apical region of the surface epithelial cells showed a weak positive reaction (Fig. 2B). The upper cytoplasm of the surface epithelium of the caudal region of the magnum showed strong immunostaining (Fig. 2C). In the isthmus, the apical or entire cytoplasm of basal cells was densely immunolabeled (Fig. 2D). The cytoplasm of the basal cells of the surface epithelium was densely immunolabeled in the uterus (Fig. 2E), whereas the basal cells of surface epithelium of the vagina contained fine immunoreactive (ir) granules (Fig. 2F).

The immunolabeling for av β D-12 was observed in the cytoplasm of basal cells in the surface epithelium of the infundibulum, caudal region of the magnum, uterus, and vagina (Fig. 3). Those immunoreaction products showed a fine granule-like appearance. The immunoreaction products were not identified in the cephalic magnum and isthmus (data not shown).

Control sections that were stained using normal rabbit IgG did not show any staining in all tissues (Fig. 4).

Figure 1 Sections of the oviduct of a laying hen just after oviposition immunostained for av β D-3; (A) infundibulum, (B) cephalic region of magnum, (C) caudal region of magnum, (D) isthmus, (E) uterus, and (F) vagina. The upper region of the cytoplasm of basal cells in the surface epithelium of the cephalic region of magnum, isthmus, uterus, and vagina and the lower cytoplasm of the caudal region of the magnum contain immunoreaction products (long arrows). The tubular gland cells of the magnum (cephalic and caudal regions) and isthmus are weakly stained (short arrows). L, lumen; E, surface epithelium; LP, lamina propria; TG, tubular glands. Scale bars, 20 μ m.

Table 1 summarizes the results of immunostaining for av β Ds in the oviductal surface epithelium. Each oviductal segment expressed immunolabeling for two or three types of av β Ds.

Western blot of av β D-3, -11, and -12 in the uterus

Western blot analysis for av β D-3, -11, and -12 in the uterus mucosal tissue showed a single band of immunoreaction products at 18, 19, and 34 kDa respectively. No specific bands were observed in the control staining using preabsorbed antibodies or normal rabbit IgG (Fig. 5A–C).

Changes in the density of av β D-3, -11, and -12 in the uterus during eggshell formation

The changes in the density of the immunoreaction products of the three av β Ds in the uterus during eggshell formation are shown in Fig. 6. The density of the immunoreaction products of av β D-3 and -12 in the surface epithelium was decreased after the egg entered the uterus, and those of 10 h group was significantly

Figure 2 Sections of the oviduct of a laying hen just after oviposition immunostained for av β D-11; (A) infundibulum, (B) cephalic region of magnum, (C) caudal region of magnum, (D) isthmus, (E) uterus, and (F) vagina. Some of the surface epithelial cells of each segments shows immunolabeling for av β D-11 in the whole cytoplasm except for the cephalic region of the magnum, where the immunoreaction products are located at the apical region of the cytoplasm. Arrows show immunoreaction products. See Fig. 1 for other abbreviations. Scale bars, 20 μ m.

smaller than 0 h group (Fig. 6A and C). In contrast, the density of av β D-11 immunoreaction products did not show significant difference among 0, 6, and 10 h groups (Fig. 6B).

Identification of av β Ds in the eggshell membrane and eggshell

In the eggshell membrane, immunolabeling of av β D-3 was detected on the surface of fibers in the outer layer of the membrane (Fig. 7), whereas no immunolabeling was detected in case of av β D-11 and -12 (data not shown).

Figures 8 and 9 show the results of western blot to confirm the presence of av β D proteins in the extramineral and intramineral extracts of the eggshell matrix proteins respectively. Specific bands were obtained at 23 and 25 kDa for av β D-3, 8 kDa for av β D-11, and 34 kDa for av β D-12 in both extramineral and intramineral extracts.

Discussion

We are reporting that ir-av β Ds were localized in hen oviduct and were identified in the eggshell membrane and eggshell. Significant findings were: 1) the surface

epithelial cells of all oviductal segments contained ir-av β D-3, -11, and/or -12, 2) the density of ir-av β D-3 and -12 in the uterus was significantly decreased in association with entering of the egg into this segment, and 3) eggshell membrane and eggshell contained ir-av β D-3, -11, and -12. Western blot analysis using proteins isolated from the uterus mucosa showed a single band for av β D-3, -11, and -12 by corresponding antibodies, but not by preabsorbed antibodies or normal rabbit IgG. These results suggest that the antibodies specifically recognized the corresponding av β Ds.

The immunoreaction products of av β D-3, -11, and -12 were located in the basal cells of surface epithelium of all segments, and additionally those of av β D-3 were in the tubular gland cells of the magnum and the isthmus. We have identified the mRNA expression of av β D-1 to -3 in the surface epithelium of the hen vagina (Ohashi *et al.* 2005). The current results tend to confirm that the surface epithelium is the major site where av β Ds are synthesized in hen oviduct. Quayle *et al.* (1998), who examined gene expression and immuno-localization of human defensin-5 (HD-5 now known as DEFA5) in human female reproductive tract, localized the ir-DEFA5 in the columnar cells of the surface epithelium of fallopian tubes, endometrium, and endocervix. It is likely a common feature among mammals and birds that the surface epithelial cells of female reproductive tract express β -defensin proteins.

A variety of microorganisms exist in the cloaca and may ascend the oviduct through the vagina in hens. The hen reproductive tract is the site of infectious *Salmonella* organisms (Chappell *et al.* 2009), which may invade the surface epithelium or subepithelial tissues (Takata *et al.* 2003). We reported that mRNA of 11 types of av β Ds including av β D-3, -11, and -12 was expressed in the

Figure 3 Sections of the oviduct of a laying hen just after oviposition immunostained for av β D-12; (A) infundibulum, (B) caudal region of magnum, (C) uterus, and (D) vagina. The upper region of the cytoplasm of basal cells in the surface epithelium contains immunoreaction products (arrows). See Fig. 1 for other abbreviations. Scale bars, 20 μ m.

Figure 4 Sections of the oviduct of laying hens just after oviposition stained with normal rabbit IgG (control staining); (A) infundibulum, (B) cephalic region of magnum, (C) caudal region of magnum, (D) isthmus, (E) uterus, and (F) vagina. No staining is observed. See Fig. 1 for other abbreviations. Scale bars, 20 μ m.

mucosal tissue throughout the oviduct (Abdel Mageed *et al.* 2008). The ir-av β D-3, -11, and -12 observed in this study confirmed that those av β Ds were synthesized in the oviduct. However, the protein amount of synthesized av β D-3 in the infundibulum and av β D-12 in the magnum and isthmus is likely negligible in healthy hens because their immunoreaction products were not identified. Previous reports suggested that, in the chicken oviduct, various antimicrobial peptides or proteins such as peroxidase, lysozyme, ovotransferrin, β -N-acetylglucosaminidase, and histone proteins were synthesized (Ahlborn *et al.* 2006, Silphaduang *et al.* 2006, Yoshimura *et al.* 2006). The av β Ds synthesized in the oviduct may participate in the formation of the innate immune system in this organ in combination with other antimicrobial peptides and proteins. Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns. We have identified the expression of six types of TLRs including TLR4 in the hen oviduct

(Ozoe *et al.* 2009). TLR4 recognizes LPS of Gram-negative bacteria such as *Salmonella* organisms. We also found that i.v. injection of hens with LPS caused an increase in the mRNA expression of av β D-3, -11, and -12 (Abdel Mageed *et al.* 2008). It is assumed that synthesis of av β Ds is enhanced in response to recognition of microorganisms by TLRs.

The density of the ir-av β D-3 and -12 in the surface epithelium of the uterus was significantly decreased when the egg entered this part of the oviduct. This result suggests the possibility that they were secreted from the epithelial cells. During the egg formation, uterus fluid containing organic substances and salts is secreted from the uterus mucosa, and the egg surrounded by eggshell membrane takes up this fluid before calcification (Johnson 1999). The av β Ds secreted by the uterus cells may be contained in the fluid. The eggshell membrane consisted of a fibrous network. The surface of the fibers was immunopositive for av β D-3, although the body of the fibers was not positive for av β D-3, -11, and -12 immunoreaction. It is suggested that av β D-3 secreted by uterus surface epithelium into the uterus fluid attached to the surface of eggshell membrane fibers at early phase when the egg takes up the uterus fluid. Western blot examination of the eggshell indicated that the eggshell also contained av β D-3, -11, and -12 proteins. Those av β Ds secreted from the uterus mucosa into the uterus fluid are likely to be incorporated in the shell matrix. Although the changes in the density of av β D-11 in the uterus surface epithelium were not significant, it is possible that av β D-11 could be involved in the secretion because eggshell contained that protein.

Western blot analysis of the eggshell showed two different-sized bands (23 and 25 kDa) that were higher than uterus av β D-3 (18 kDa). The two different forms of av β D-3 might be appeared by binding of some residues to the tissue form of av β D-3 during the process of secretion. Yudin *et al.* (2005) reported that carbohydrate residues were added to the defensin molecules to enhance their activity. On the other hand, av β D-11 in the eggshell showed a band smaller than the tissue form, indicating that some splicing might be caused in the tissue form of av β D-11 during secretion. In contrast, the molecular size of av β D-12 was similar to the tissue form, indicating that neither splicing nor adding of residues may occur in the tissue form.

Table 1 Summary of the distribution of immunoreactive av β D-3, -11, and -12 in the surface epithelium of different segments of the hen oviduct.

Types of av β Ds	Infundibulum	Magnum			Isthmus	Uterus	Vagina
		Cephalic	Caudal				
av β D-3	ND	+	+		+	+	+
av β D-11	+	+	+		+	+	+
av β D-12	+	ND	+		ND	+	+

+, immunoreaction products were found. ND, no products were detected.

Figure 5 Western blotting for av β D-3 (A), -11 (B), and -12 (C) proteins from the mucosal tissue of the uterus just after oviposition. Lane 3 was immunostained by av β D antibodies, whereas lanes 1 and 2 were stained using preabsorbed antibody and normal rabbit IgG in place of av β D antibodies respectively. Arrows show the specific bands for immunoreactive av β Ds. Specific bands were identified at 18, 19, and 34 kDa for av β D-3, -11, and -12 respectively.

Previous studies showed that the components of the eggshell membrane and eggshell exhibit antimicrobial activities (Poland & Sheldon 2001, Mine *et al.* 2003). More recently, antimicrobial proteins including β -N-acetylglucosaminidase, lysozyme, and ovotransferrin were identified in the eggshell membrane (Ahlborn *et al.* 2006). Properties of antimicrobial proteins such as L-type lysozyme, ovotransferrin, ovocalyxin-36 and c-type lectin-like proteins are also reported in the eggshell matrix (Gautron *et al.* 2006, Wellman-Labadie *et al.* 2008). The av β Ds in the eggshell membrane and eggshell matrix may form the defense system of the egg surface in combination with other antimicrobial proteins.

In conclusion, we suggest that the surface epithelial cells are the site where av β D-3, -11, and -12 proteins are present in the oviduct, and the av β Ds in the uterus are likely to be secreted and incorporated in the eggshell membrane and eggshell during shell formation. These av β Ds may play roles in the host innate immune system in the oviduct and defense of the eggshell membrane and eggshell.

Materials and Methods

Experimental birds

White Leghorn hens of $\sim$ 400-day-old age and laying five or more eggs in a sequence were used. They were kept in individual cages and provided with feed and water *ad libitum* under a light regimen of 14 h light:10 h darkness. The birds were divided into three groups and oviductal samples were collected at different times of the ovulatory cycle; 0 h group (just after oviposition; corresponding to the time before the entrance of ovum into the oviduct, namely just before ovulation), 6 h group (6 h after oviposition; just after the entrance of egg into the uterus), and 10 h group (10 h after oviposition; $\sim$ 4 h after the entrance of eggs in the uterus). They were killed under anesthesia with sodium pentobarbital (Abbott Laboratories) before collecting the oviducts. Handling of birds was done in accordance with the regulations of Hiroshima University for animal experiments.

Antibodies to Ds

Rabbit anti-av β D-3 and anti-av β D-12 antiserum were produced in rabbits using synthetic peptides and have been used in the previous studies (Yoshimura *et al.* 2007, Subedi *et al.* 2008). Antiserum to av β D-11 was prepared by immunization of rabbits with KLH-conjugated synthetic peptide. The sequences of the synthetic peptides were as follows: av β D-3, RFPHI AIGKC ATF; av β D-11, CPKPF AAFGT; and av β D-12, GPDSC NHDRG LCRVG NCNPG EYLAK YCFEP VILCC KPLSP TPTKT. The sequences of those peptides corresponded to their specific sequence (Xiao *et al.* 2004). The IgGs in those antiserum and normal rabbit serum (for control staining) were purified using HiTrap affinity Protein G

Figure 6 Changes in the density of the immunoreactive av β D-3 (A), -11 (B), and -12 (C) in the surface epithelium of the uterus during the egg formation (ratio of immunopositive area). The temporal status of the ovum of groups 0, 6, and 10 h after oviposition corresponds to just before ovulation, just after entering the uterus, and 4 h after entering the uterus respectively. Values are mean $\pm$ S.E.M. ($n=4$). Values with different letters are significantly different ($P<0.05$).

Figure 7 Micrograph of the eggshell membrane immunostained for avβD-3. Note that the surface of the fibers at the outer layer is densely stained (arrows). Control stained with normal rabbit IgG shows no staining. Scale bars, 20 μm.

HP column (GE Healthcare Bio-Sciences AB, Björkgatan 30, Uppsala, Sweden). Preabsorbed antibodies were prepared by incubating the antibody with excess of its corresponding peptide overnight at 4 °C.

Immunohistochemistry for avβDs in the oviduct and eggshell membrane

Immunostaining

The oviductal tissues of 0 h group were examined to identify the general localization of avβDs in the entire oviduct. Changes in the localization of avβDs in the uterus were examined in 0, 6, and 10 h groups ($n=4$ each). Tissues of the tubular region of the infundibulum, magnum (cephalic and caudal regions), isthmus, uterus, and vagina were fixed in freshly prepared 4% (w/v) paraformaldehyde in PBS and processed for paraffin sections (4 μm). The eggshell membranes were stripped from the eggshell of fresh eggs ($n=4$), and processed for paraffin sections in a same manner.

Sections were deparaffinized and rehydrated before they were autoclaved for 10 min in 0.1 mol/l citric acid (pH 6.8) for antigen activation. Sections were then cooled and washed in PBS (3×5 min). The immunohistochemical staining was performed using anti-avβD-3, -11, and -12 antibodies, and Vectastain ABC kit (Vector Lab., Inc., Burlingame, CA, USA) according to the manufacturer's instructions. Briefly, sections were incubated with a blocking solution (normal goat serum, 15 μl/ml in PBS) for 1 h in a humid chamber at room

Figure 8 Western blotting for avβD-3 (A), -11 (B), and -12 (C) proteins from the extramineral extract of eggshell. Specific bands are identified at 23 and 25 kDa for avβD-3, 8 kDa for avβD-11, and 34 kDa for avβD-12 (arrows). Lanes 3 of A–C were immunostained by specific antibodies, whereas lanes 1 and 2 were incubated with preabsorbed antibodies or normal rabbit IgG (control staining). NS, nonspecific bands that were found in control staining.

Figure 9 Western blotting for avβD-3 (A), -11 (B), and -12 (C) proteins from the intramineral extract of eggshell. Specific bands are identified at 23 and 25 kDa for avβD-3, 8 kDa for avβD-11, and 34 kDa for avβD-12. For more explanation, refer to Fig. 8.

temperature. They were then incubated for 4 h at room temperature with one of the antibodies to avβDs or normal rabbit IgG (for control staining) diluted at a concentration of 20 μg/ml using the blocking solution. Sections were then washed in PBS (5 min×3) and incubated with biotinylated goat anti-rabbit IgG and avidin–biotin peroxidase complex for 1 h each. After washing in PBS (5 min×3), the immunoreaction products were visualized using 0.02% (w/v) 3,3'-diaminobenzidine tetrahydrochloride and 0.005% (v/v) hydrogen peroxide in 0.05 mol/l Tris–HCl, pH 7.6. Sections were then counterstained with hematoxylin, dehydrated in ascending grades of ethanol, cleared, and covered.

Image analysis

Sections of the uterus immunostained for avβDs were examined under a light microscope with image analysis software (Image-Pro Plus, Media Cybernetics, Silver Springs, MD, USA). The immunopositive area and total measured area in the surface epithelium were analyzed, and the ratio of positive area to the total measured area was obtained. Three different regions in one tissue were analyzed and the mean value was used for the value of one tissue.

SDS-PAGE and western blotting for Ds in the uterus and eggshell

Sample preparation

Uterus mucosa protein. Uterus mucosal tissues of healthy birds of 0 h group were collected and homogenized in five times volume of homogenization buffer consisting of 0.02 mol/l Tris–HCl, pH 7.4, 0.15 mol/l NaCl, 0.005 mol/l EDTA, 1% (v/v) Triton X-100, 10% (w/v) glycerol, 0.1% (w/v) SDS, and 0.001 mol/l phenylmethylsulfonylfluoride (PMSF). The samples were centrifuged at 12 000 g for 20 min. The supernatant was collected, and the proteins were precipitated by 40% (w/v) ammonium sulfate and centrifuged at 9000 g for 20 min. The protein precipitates were dissolved in water at a concentration of 1.5 μg/μl and used as the protein samples. The protein concentration was measured using protein assay reagent (Bio-Rad Lab) as described by the manufacturer.

Eggshell matrix protein. The proteins in the eggshell matrix were extracted as described by *Hincke et al. (2000)* and *Gautron et al. (2001)*. Briefly, the eggshells without eggshell membrane were collected from fresh eggs ($n=4$; ten eggs were pooled for one sample). They were rinsed in saline containing 0.001 mol/l PMSF. The eggshells were then air-dried and ground into fine powder. The powder (25 g) was continuously stirred at room temperature in 100 ml of 4 mol/l guanidine-HCl (pH 7.4) for 4 days at 4 °C. The mixture was then centrifuged at 12 000 g to separate the supernatant (extramineral extracts) and precipitate. The precipitate fractions were further demineralized with 0.5 mol/l EDTA, 0.05 mol/l Tris-HCl (pH 7.4) for 1 h, and mixed with 200 ml of 4 mol/l guanidine-HCl (pH 7.4) for 1 day with continuous shaking at 4 °C. They were then centrifuged at 12 000 g , and the supernatant was collected as intramineral extracts.

The extramineral and intramineral extracts were filtered through 0.45 μ m cellulose acetate filter (Toyo Roshi Kaisha Ltd, Tokyo, Japan), followed by isolation of proteins using Sep-Pak cartridge (Waters Corp., Milford, MA, USA). The eluted fractions were freeze-dried, and the protein concentration was adjusted to 1 mg/ml using PBS.

Tricine-SDS-PAGE

The samples were separated by Tricine-SDS-PAGE (16% (w/v) polyacrylamide separating gel and 4% (w/v) stacking gel) as described by *Schägger (2006)* with minor modifications. Briefly, samples were dissolved in sample buffer composed of 30% (v/v) glycerol, 5% (v/v) mercaptoethanol, 4% (w/v) SDS, 0.15 mol/l Tris-HCl, pH 7.0, and 0.06% (w/v) bromophenol blue, and boiled for 10 min. An aliquot of each sample (10 μ g protein) was loaded onto gels and run at 80 V in the stacking gel and at 150 V in the separating gel.

Western blotting

After SDS-PAGE, the proteins in the gel were electrophoretically transferred onto the nitrocellulose membrane (PALL Gelman Laboratory, Ann Arbor, MI, USA) at 350 mA for 60 min. The membrane was washed with western buffer (0.02 M Tris-HCl, pH 7.4, 0.15 mol/l NaCl, 0.5% (w/v) Tween 20, and 0.05% (w/v) BSA) for 15 min (5 min $\times$ 3) and incubated with 5% (w/v) casein milk (Roche) solution in western buffer (for 1 h for uterus mucosa tissue samples; 2 h for eggshell samples). The membranes blotted with uterus mucosa proteins were incubated with av β D-3, -11, or -12 antibodies diluted at a concentration of 10 μ g/ml in 1% (w/v) casein milk diluted in Can Get Signal Immunoreaction Enhancer Solution 1 for primary antibody (Toyobo Co., Ltd, Osaka, Japan) for 1 h at room temperature. The membranes blotted with eggshell proteins were incubated for 2 h with antibodies to av β D-3 or -11 (10 μ g/ml) or for 4 h with antibody to av β D-12 (20 μ g/ml). The membrane was then washed in western buffer for 30 min (10 min $\times$ 3) before incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (Assay Designs, Inc., Ann Arbor, MI, USA) diluted at 1:5000 in 1% (w/v) casein milk in Can Get Signal Immunoreaction Enhancer Solution 2 for second antibody (Toyobo Co., Ltd) for 1 h at room temperature.

The membrane was washed with western buffer for 30 min (10 min $\times$ 3 times), and the immunoprecipitates on the membrane were visualized by incubating in a reaction mixture composed of 0.017% (w/v) 5-bromo-4-chloro-3-indolyl phosphate disodium salt (Sigma-Aldrich, Inc.) and 0.45% (w/v) nitro blue tetrazolium (Nacalai Tesque, Inc., Kyoto, Japan) in substrate buffer (0.1 mol/l Tris-HCl (pH 9.5), 0.1 mol/l NaCl, and 0.5 mol/l MgCl₂). The membrane was finally washed in running water to stop the reaction.

For the control staining, the first antibodies were replaced with normal rabbit IgG or preabsorbed antibodies.

Statistical analysis

The results were expressed as mean $\pm$ S.E.M. of the ratio of immunopositive area to total measured area (av β Ds immunohistochemistry in the uterus). The significance of differences was examined using a one-way ANOVA, followed by Duncan's multiple range test. A P value of <0.05 was considered statistically significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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