Induction of avian β -defensins by CpG oligodeoxynucleotides and proinflammatory cytokines in hen vaginal cells *in vitro*

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Abstract

Immune function in the vagina of hen oviduct is essential to prevent infection by microorganisms colonizing in the cloaca. The aim of this study was to determine whether CpG oligodeoxynucleotides (CpG-ODN) stimulate the expression of avian β -defensins (AvBDs) in hen vaginal cells. Specific questions were whether CpG-ODN affects the expression of AvBDs and proinflammatory cytokines and whether the cytokines affect AvBDs expression in vaginal cells. The dispersed vaginal cells of White Leghorn laying hens were cultured and stimulated by different doses of lipopolysaccharide (LPS), CpG-ODN, interleukin 1 β (IL1 β), or IL6. The cultured cell population contained epithelial cells, fibroblast-like cells, and CD45-positive leukocytes. The immunoreactive AvBD3, -10, and -12 were localized in the mucosal epithelium in the section of the vagina. The expression of AvBDs, IL1 β , and IL6 was analyzed by quantitative RT-PCR. RT-PCR analysis showed the expression of AvBD1, -3, -4, -5, -10, and -12 in the cultured vaginal cells without stimulation. Toll-like receptors (TLRs) 4 and 21, which recognize LPS and CpG-ODN respectively and IL1 and IL6 receptors (IL1R1 and IL6R) were also expressed in them. The expression of IL1 β , IL6, and AvBD10 and -12 was upregulated by LPS, whereas only IL1 β and IL6 were upregulated by CpG-ODN. IL1 β stimulation upregulated AvBD1 and -3 expression, whereas IL6 stimulation did not cause changes in AvBDs expression. These results suggest that CpG-ODN derived from microbes upregulates the expression of IL1 β and IL6 by interaction with TLR21 and then IL1 β induces AvBD1 and -3 to prevent infection in the vagina.

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Introduction

In hens, the vagina opens to the cloaca where various microorganisms colonize. The host defense functions of the vagina play essential roles to prevent infection by these microorganisms ascending the oviduct. Toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns initiate innate immune responses. To date, ten TLRs have been identified in chickens (Brownlie & Allan 2011). Among them, TLR4 recognizes lipopolysaccharide (LPS), which is a major component of the outer membrane of Gram-negative bacteria. CpG oligodeoxynucleotide (CpG-ODN) containing unmethylated CpG motifs is conserved in the genomic DNA in bacteria (Krieg *et al.* 1995, Wagner 1999). The CpG-ODN is recognized by TLR9 in mammals (Hemmi *et al.* 2000, Bauer *et al.* 2001), whereas TLR21 acts as a functional homolog to mammalian TLR9 in the recognition of CpG-ODN in chickens (Brownlie *et al.* 2009). The expression of proinflammatory cytokines, interferon (IFN) γ , and nitric oxide was found to be induced in response to CpG-ODN in mononuclear cells and splenocytes in chickens (He *et al.* 2003, Patel *et al.* 2008). However, it is unknown whether an immune

response is induced by CpG-ODN in the mucosal tissue of hen vagina.

Defensins, antimicrobial peptides, are cysteine-rich cationic peptides (Selsted & Ouellette 2005). The avian β -defensins (AvBDs) are characterized by six cysteine residues, and 14 AvBDs genes have been identified in chickens (Lynn *et al.* 2007). They have the potential to kill a wide spectrum of microorganisms, including Gram-positive and Gram-negative bacteria, fungi, and yeasts (Yang *et al.* 2002, Sugiarto & Yu 2004). Proinflammatory cytokines such as interleukin 1 β (IL1 β) and IL6 also play important roles in innate and adaptive immune systems and induce inflammatory responses in infected tissue (Staeheli *et al.* 2001, Ferro *et al.* 2004). IL1 β was shown to enhance the production of immune-related molecules, such as nitric oxide, acute-phase proteins, cytokines, and chemokines (Arend *et al.* 2008). IL6 has multiple functions, such as stimulation of antibody synthesis by B cells (Okada *et al.* 1983) and the differentiation of monocytes from dendritic cells to macrophages (Chomarat *et al.* 2000). We have reported that the injection of birds with LPS enhanced the expression of both AvBDs and proinflammatory cytokines, IL1 β and IL6, in the vagina

(Abdel Mageed *et al.* 2008, Nii *et al.* 2011). In mammals, there are reports that β -defensin expression was upregulated by IL1B in keratinocytes, and in corneal and uterus epithelium (Liu *et al.* 2002, McDermott *et al.* 2003, Shin *et al.* 2004, Pioli *et al.* 2006). If CpG-ODN was shown to enhance AvBDs expression, this would provide a novel understanding of the process of AvBDs induction in response to bacterial components in the vagina. For further determination of the mechanism by which AvBDs expression is regulated downstream of CpG-ODN stimulation, the role of proinflammatory cytokines that may be expressed by CpG-ODN in AvBDs expression should also be examined.

The aim of this study was to determine whether CpG-ODN leads to an increase in the AvBDs expression in hen vagina. In experiment 1, the effects of LPS and CpG-ODN on the expression of *IL1B*, *IL6*, and AvBDs in cultured vaginal cells were examined. This experiment examined whether CpG-ODN induces the expression of AvBDs and proinflammatory cytokines and whether there are differences in the effects of their induction between CpG-ODN and LPS. Then, in experiment 2, the effects of IL1B and IL6 on the AvBDs expression in those cells were examined. The induction of AvBDs by these proinflammatory cytokines was analyzed to know the possibility that CpG-ODN induces the cytokines and then they affect AvBDs expression.

Results

Figure 1 shows the pattern of RT-PCR products of AvBDs, *TLR4* and *TLR21*, and IL1 and IL6 receptors (*IL1R1* and *IL6R*) in vaginal mucosa cells cultured for 24 h. Clear bands of six AvBDs including AvBD1, AvBD3–5, AvBD10, and AvBD12 were identified (Fig. 1a). Thus, the changes in the expression of these six AvBDs in response to LPS, CpG-ODN, IL1B, and IL6 were

examined in experiments 1 and 2. The PCR products of *TLR4* and *TLR21*, and *IL1R1* and *IL6R*, were also identified (Fig. 1b and c). The cultured cell population contained epithelial cells including the cells positive for Alcian blue and Periodic acid-Schiff reaction (AB-PAS), elongated fibroblast-like cells, and CD45-positive leukocytes (Fig. 1d and e). The immunoreaction products for AvBD3, -10, and -12 were identified in the mucosal epithelium in the section of the vagina (Fig. 2a, b, and c), but the negative control staining using normal rabbit IgG did not show any immunoreaction products (Fig. 2d).

Experiment 1: effects of LPS and CpG-ODN on the expression of *IL1B*, *IL6*, and AvBDs in vaginal cells

Figure 3 shows the changes in the expression of *IL1B*, *IL6*, and AvBDs in the vaginal cells stimulated by different doses of LPS. The *IL1B* expression was significantly higher at ~17-fold in the 10^2 ng/ml LPS group and was also higher in the 10^3 and 10^4 ng/ml LPS groups compared with that in the control (0 ng/ml LPS) (Fig. 3a). The *IL6* expression was significantly upregulated by 10^4 ng/ml LPS (Fig. 3b). The expression of AvBD1, -3, -4, and -5 was not affected by LPS stimulation (Fig. 3c, d, e, and f). However, the expression of AvBD10 was significantly higher at approximately fourfold in the 10^2 ng/ml LPS group compared with that in the control, but this was not caused by a higher dose of LPS (Fig. 3g). AvBD12 expression was also significantly higher in the 10^4 ng/ml LPS group than in the control (Fig. 3h).

The effects of different doses of CpG-ODN on the expression of *IL1B*, *IL6*, and AvBDs are shown in Fig. 4. The expression of *IL1B* and *IL6* was upregulated ~500- and 8-fold respectively in the 1 and 10 μ g/ml CpG-ODN groups (Fig. 4a and b). No significant effects of CpG-ODN on AvBDs expression were identified (Fig. 4c, d, e, f, g, and h).

Figure 1 Profile of RT-PCR products for 14 avian β -defensins (AvBDs) (a), *TLR4* and *TLR21* (b), receptors of IL1 and IL6 (*IL1R1* and *IL6R*) (c), and cell type identification (d and e) in the cultured vaginal cells. The PCR products of AvBDs, *TLRs*, and IL receptors were electrophoresed on 2% agarose gel containing ethidium bromide. M=100 bp DNA size marker. Micrographs of (d) and (e) show the cultured cells stained by Alcian blue (AB) and Periodic acid-Schiff reaction (PAS), and immunostained by anti-CD45 antibody respectively. In the micrograph of (d), AB-positive (short arrows) and PAS-positive (long arrows) epithelial cells and fibroblast-like cells (arrow heads) are observed. In the micrograph of (e), CD45-positive cells are indicated by arrows. Scale bars represent 50 μ m.

Figure 2 Micrographs of the vagina immunostained for AvBD3, -10, and -12. Immunoreaction products for AvBD3 (a), AvBD10 (b), and AvBD12 (c) are localized in the mucosal epithelium (arrows). Control staining using normal rabbit IgG in place of primary antibodies shows no positive reaction product (d). E, mucosal epithelium; L, lamina propria. Scale bars represent 30 μ m.

Experiment 2: effects of IL1B and IL6 on the expression of AvBDs in vaginal cells

Figure 5 shows the effects of different doses of IL1B on the expression of AvBDs. The expression of AvBD1 was significantly increased ~ 2.5 -fold by 10^2 or 10^3 ng/ml IL1B (Fig. 5a). The AvBD3 expression showed approximately fivefold change due to 10^3 ng/ml IL1B (Fig. 5b). The expression of AvBD4, -5, -10, and -12 was not changed by IL1B (Fig. 5c, d, e, and f).

The effects of IL6 on the AvBDs expression are shown in Fig. 6. The expression of AvBD1, -3, -4, -5, and -10 was not affected by 10^2 or 10^3 ng/ml IL6 (Fig. 6a, b, c, d, and e). The AvBD12 expression was significantly higher in the 10^3 ng/ml IL6 group than in the 10^2 ng/ml IL6 group, whereas the expression of both groups was not different from that of the control (0 ng/ml IL6 group) (Fig. 6f).

Discussion

We report that the expression of proinflammatory cytokines and AvBDs is induced in response to LPS and CpG-ODN in cultured mucosal cells of the vagina and that IL1B also upregulates AvBDs expression. Significant findings were as follows: i) stimulation of the vaginal cells by LPS enhanced the expression of IL1B and IL6 and also AvBD10 and -12, whereas CpG-ODN upregulated only IL1B and IL6. ii) IL1B upregulated the expression of AvBD1 and -3, but IL6 did not show significant effects on AvBDs expression by the vaginal cells. Previous studies revealed that the expression of AvBD1–5 and AvBD8–12 was identified in fresh vaginal tissue (Abdel Mageed *et al.* 2008), whereas the expression of AvBD1, AvBD3–5, and AvBD9–14 was higher than that of AvBD2 and AvBD6–8 in cultured

oviductal epithelial cells obtained from the isthmus (Ebers *et al.* 2009). The current study showed clear RT-PCR products of AvBD1, -3, -4, -5, -10, and -12 in the cultured vaginal cells. This result partially supports the findings of Ebers *et al.* (2009) and suggests that the expression of these six AvBDs in the vaginal cells is maintained even after cell culture for 24 h. The cultured cell population involved the epithelial cells, fibroblast-like cells, and leukocytes expressing CD45, a leukocyte common antigen (Symons *et al.* 1999). Immunoreactive AvBD3, -10, and -12 were localized in the mucosal epithelium, supporting our previous reports that localized immunoreactive AvBD3, -11, and -12 in the mucosal epithelium of the hen vagina (Yoshimura *et al.* 2007, Abdel Mageed *et al.* 2009). Although the cells immunoreactive for AvBD1 have not been examined because of the lack of its antibody, many of the AvBD proteins are likely to exist in the mucosal epithelium in the vagina.

The expression of IL1B and IL6 was upregulated by LPS and CpG-ODN in the vaginal cells (Figs 3 and 4). Previous studies reported that TLR1 type 1, 2–5, 7, 15, and 21 were expressed in chicken vaginal tissues (Ozoe *et al.* 2009, Michailidis *et al.* 2011). Among these TLRs, TLR4 recognized LPS, whereas TLR21, an avian-specific TLR, recognized unmethylated CpG motifs as TLR9 in mammals (Brownlie & Allan 2011). The current study further confirmed that TLR4 and 21 expression was detected in cultured vaginal cells (Fig. 1b). These two TLRs play important roles in the defense against *Salmonella* infection because susceptibility to *Salmonella enteritidis* is closely related to the responsiveness of three TLRs, namely, TLR4, TLR21, and TLR2 type 1 (Gou *et al.* 2012). It is likely that the upregulation of IL1B and IL6 expression by LPS is caused by an interaction of LPS

Figure 3 Changes in the expression of *IL1B*, *IL6*, and *AvBDs* in the cultured vaginal cells in response to different doses of LPS. The cultured vaginal cells were stimulated by 0–10⁴ ng/ml LPS, and the relative expression of *IL1B* and *IL6* (a and b) and *AvBD1*, 3, 4, 5, 10, and 12 (c, d, e, f, g, and h) was examined by real-time PCR. Values are mean $\pm$ S.E.M. ($n=6$). ^{a,b,c}The values with different letters are significantly different ($P<0.05$).

with TLR4 in the vaginal cells. The induction of these proinflammatory cytokines by LPS supports the results of our previous *in vivo* study in the vagina (Ozoe *et al.* 2009). In addition, it is assumed that the enhancement of *IL1B* and *IL6* expression by CpG-ODN is mediated by TLR21 expressed in the vaginal cells.

The expression of *AvBD10* and -12 in the vaginal cells was elevated by exposure of cells to LPS at 10² and 10⁴ ng/ml respectively (Fig. 3). However, the *AvBD10* expression was not increased by 10³ or 10⁴ ng/ml LPS. Although the reason why higher dose of LPS did not elevate *AvBD10* expression is not known, it may be toxic for the vaginal cells, and disturb the expression of some

AvBDs. We have observed that expression of *AvBD1*, -7, and -12 in the ovarian follicles were increased by injection of birds with LPS at 1 mg/kg BW, but not at 2 mg/kg BW, in laying hens (Subedi *et al.* 2007). In contrast, CpG-ODN did not show any significant effects on *AvBDs* expression (Fig. 4). In the downstream of TLRs, signal molecules involved in Myd88-dependent or -independent pathways regulate transcriptional factors such as nuclear factor- κ B (NF κ B) and activator protein 1 (AP1), leading to the initiation of transcription in the nucleus (Riley & Nelson 2010). The release of human β -defensin (hBD) 2 was found to be controlled by phosphoinositide 3 kinase (PI3K) and NF κ B, whereas

Figure 4 Changes in the expression of *IL1B*, *IL6*, and *AvBDs* in the cultured vaginal cells in response to different doses of CpG-ODN. The cultured vaginal cells were stimulated by 0–10 µg/ml CpG-ODN, and the relative expression of *IL1B* and *IL6* (a and b) and *AvBD1*, -3, -4, -5, -10, and -12 (c, d, e, f, g, and h) was examined. Values are mean $\pm$ S.E.M. ($n=6$). ^{a,b}The values with different letters are significantly different ($P<0.05$).

hBD 3 was triggered via the c-Jun N-terminal kinase (JNK)-AP1 pathway in human lung epithelium (Scharf *et al.* 2012). Different TLRs may modulate different molecules that activate transcriptional factors, such as IFN regulatory factor 3 (*IRF3*), *NFκB*, and *AP1* (Rhee

2011). Andersen *et al.* (2006) reported that, in cervical epithelial cells, *IL8* expression was upregulated by CpG-DNA or poly(I:C), the ligands of TLR9 and TLR3, respectively, whereas IFN β and CC chemokine were induced by poly(I:C) but not by CpG-DNA. Thus, it is

Figure 5 Changes in the expression of AvBDs in the cultured vaginal cells in response to different doses of IL1B. The cultured vaginal cells were stimulated by 0–10³ ng/ml recombinant chicken IL1B and the relative expression of AvBD1, -3, -4, -5, -10, and -12 was examined (a–f). Values are mean $\pm$ S.E.M. ($n=6$). ^{a,b}The values with different letters are significantly different ($P<0.05$).

assumed that the intracellular signaling pathways of LPS and CpG-ODN stimulation for transcriptional regulation of AvBDs and *IL1B* and *IL6* are different. LPS might activate a pathway that induces both AvBD10 and -12 as well as *IL1B* and *IL6*, whereas CpG-ODN might activate only the pathway for *IL1B* and *IL6*.

The expression of AvBD1 and -3 in the vaginal cells was upregulated by exposure to IL1B but not IL6 (Figs 5 and 6). We have reported that, in the theca of hen ovarian follicles, IL1B enhanced the expression of AvBD12, but IL6 did not show significant effects on its expression (Abdelsalam *et al.* 2012). In humans, hBD expression was induced in keratinocytes and corneal epithelial cells by IL1B (Liu *et al.* 2002, McDermott *et al.* 2003). Harder *et al.* (2000) reported that IL1B and TNF α , but not IL6, induced hBD 2 in human respiratory epithelia. Thus, IL1B, but not IL6, likely plays a role in the induction of AvBDs in the hen vagina as in other

tissues of hens and mammals. In this study, the expression of *IL1R1* and *IL6R* was identified in cultured vaginal cells (Fig. 1c). Thus, it is suggested that IL1B synthesized in response to bacterial components such as LPS and CpG-ODN stimulated AvBD1 and -3 expression in an autocrine and/or paracrine manner by interaction with *IL1R1* in the vaginal cells. Exposure of the vaginal cells to LPS increased the expression of AvBD10 and -12 (Fig. 3). The intracellular pathway or transcriptional regulation of AvBDs may differ between IL1B and LPS because they induced different AvBDs; namely, IL1B-induced AvBD1 and -3, whereas LPS-induced AvBD10 and -12.

AvBDs display a wide range of microbicidal or microbiostatic activities against Gram-negative and Gram-positive bacteria as well as fungi (van Dijk *et al.* 2008). Although it remains unknown whether the target microorganisms differ among the different AvBDs, the

Figure 6 Changes in the expression of AvBDs in the cultured vaginal cells in response to different doses of IL6. The cultured vaginal cells were stimulated by 0–10³ ng/ml recombinant chicken IL6, and relative expression of AvBD1, -3, -4, -5, -10, and 12 was examined (a–f). Values are mean $\pm$ S.E.M. ($n=6$). ^{a,b}The values with different letters are significantly different ($P<0.05$).

defense functions against pathogens may be stronger if different AvBDs are induced when tissue is infected. Thus, the ability of vaginal cells to induce various immune molecules including proinflammatory cytokines and different AvBDs may enable the tissue to form a well-developed defense system. We have confirmed that the injection of laying or molting hens with LPS induced the expression of *IL1B*, *IL6*, and *CXCL12* chemokine in association with CD8+ and CD4+ T-cell influx, suggesting that a host defense system formed by different immune factors is activated by pathogenic agents in hen vagina (Nii *et al.* 2011). The cultured mucosal cells of the vagina consisted of different types of cells in this study, and thus some of them are assumed to synthesize proinflammatory cytokines. We assume that immune factors such as IL1B derived from those cells in the vaginal mucosa are responsible for induction of AvBDs.

In conclusion, we suggest that chicken vaginal cells are sensitive to CpG-ODN to induce proinflammatory cytokines, IL1B and IL6, probably through interaction with TLR21. The synthesized IL1B may induce AvBD1 and -3 in the vaginal cells. Stimulation of the tissue by LPS may induce not only proinflammatory cytokines but also AvBD10 and -12. The ability to induce a variety of immune molecules including proinflammatory cytokines and different AvBDs in response to pathogenic agents may enable the vaginal tissue to form a more efficient defense system against bacterial infection.

Materials and Methods

Experimental birds

White Leghorn hens (~400 days old) laying five or more eggs in a sequence were used. They were kept in individual cages under a lighting regimen of 14 h light:10 h darkness and

provided with feed and water *ad libitum*. They were killed under anesthesia with sodium pentobarbital and the oviducts were collected at 4 h after oviposition. This study was carried out in accordance with the Guidelines for Animal Experimentation, Hiroshima University, Japan.

TLR ligands and recombinant IL1B and IL6

The LPS of Southern Minnesota and synthetic class B CpG-ODN (2007) (CpG-ODN) were purchased from InvivoGen (San Diego, CA, USA), and recombinant chicken IL1B and IL6 were from AbD Serotec (Oxford, UK). The sequence of CpG-ODN was 5'-TCGTCGTTGTCGTTTTCGTT-3' (Patel *et al.* 2008). The LPS and CpG-ODN were dissolved in endotoxin-free water and kept at -20°C until use.

Cell culture of vaginal mucosa cells

Mucosal tissues collected from the middle part of the vagina were washed in sterile PBS containing 10 U/ml penicillin and 10 $\mu\text{g}/\text{ml}$ streptomycin (Cosmo Bio Co., Ltd., Tokyo, Japan). They were cut into small specimens and incubated with a mixture of 400 U/ml DNase I (Worthington Biochemical Co., Raynham, MA, USA), 320 mg/ml liberase TL (Roche Diagnostics GmbH Co.), and 1600 U/ml hyaluronidase (Nacalai Tesque Co., Kyoto, Japan) in PBS for 30 min at 37°C on a water bath. After the cells were dispensed by pipetting, they were filtrated through a stainless steel mesh and washed three times using TCM-199 culture medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% bovine serum (Biological Ind., Kibbutz Beit Haemek, Israel), 10 U/ml penicillin, and 10 $\mu\text{g}/\text{ml}$ streptomycin (Cosmo Bio Co., Ltd.). The cell viability examined by trypan blue staining was more than 90%. The separated cells were placed on six-well tissue culture plates at a density of 2.5×10^7 vial cells/well, containing 5 ml TCM-199 culture medium, and incubated in a CO_2 incubator with 5% CO_2 and 95% air at 37°C for 24 h. Then, the wells were washed to remove nonadherent and dead cells using TCM-199 culture medium.

A part of separated cell samples were also cultured on sterile cover glasses for 24 h and fixed with 10% (v/v) formalin in PBS or cold acetone. The formalin-fixed cells were stained by AB-PAS to identify the mucosal epithelial cells containing mucopolysaccharide. The cell samples fixed with acetone were used for identification of leukocytes by immunocytochemistry for CD45, a leukocyte common antigen (Symons *et al.* 1999). They were incubated overnight with mouse anti-chicken CD45 antibody (Southern Biotech, Birmingham, AL, USA) diluted at 1:500 in PBS. Then, they were incubated with biotinylated anti-mouse IgG and avidin–biotin–peroxidase complex in Vecta Stain ABC mouse IgG kit (Vector Lab., Inc., Burlingame, CA, USA) for 30 min and 1 h respectively. Immunoreaction products were visualized by incubating the samples with 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). They were dehydrated and mounted. These staining of cultured cells were repeated in duplicate.

Immunohistochemistry for AvBDs

The vaginal tissues of the experimental birds ($n=3$) were fixed in 10% (v/v) formalin in PBS and processed for paraffin sections (4 μm in thickness) that were air-dried in MAS-coated pre-cleaned slides (Matsunami Glass, Inc., Osaka, Japan). The immunohistochemistry was performed using rabbit antibodies to AvBD3, -10, and -12 for primary antibodies that were used in our previous studies (Abdel Mageed *et al.* 2009, Abdelsalam *et al.* 2010). Vecta Stain ABC rabbit IgG kit (Vector Lab., Inc.) was used to identify the immunoreaction products. Briefly, after deparaffinization, antigen retrieval of the sections was performed by autoclaving them for 1 min in 0.1 M citric acid, pH 6.0. They were incubated with blocking solution (1.5% (vol/vol) normal goat serum in PBS) for 1 h at room temperature. Then sections were incubated overnight with antibodies to AvBD3, -10, or -12 diluted at a concentration of 20 $\mu\text{g}/\text{ml}$ followed by washing with PBS (3×5 min). The sections were then incubated with biotinylated anti-rabbit IgG and avidin–biotin–peroxidase complex for 1 h each and were washed with PBS. Immunoprecipitates were visualized by incubating the sections with DAB– H_2O_2 . The sections were dehydrated and covered and examined under a light microscope with a Nomarsky filter (Nikon Eclipse E600; Nikon, Tokyo, Japan). Control staining was carried out simultaneously in which the first antibody was replaced with normal rabbit IgG.

Stimulation of cultured cells

In experiment 1, the effects of LPS and CpG-ODN on the expression of proinflammatory cytokines and AvBDs in the vaginal cells were examined. Different doses of LPS (0 – 10^4 ng/ml) or CpG-ODN (0 – 10 $\mu\text{g}/\text{ml}$) were added to the wells containing the cells cultured for 24 h. After 3 h incubation, the expression of IL1B, IL6, and AvBD1, AvBD3–5, AvBD10, and AvBD12 was examined. We applied incubation for 3 h because the expression of IL1B, IL6, and AvBDs responded to LPS by 3 h in the theca in our previous study (Abdelsalam *et al.* 2012). In experiment 2, the effects of IL1B and IL6 on the expression of AvBDs were examined. The cultured cells were stimulated by 0 – 10^3 ng/ml IL1B or 0 – 10^3 ng/ml IL6 for 3 h. In each experiment, trials were repeated six times.

RNA isolation and cDNA preparation

Total RNA was extracted from the cultured vaginal cells using Sepasol RNA I Super (Nacalai Tesque, Inc.) in each experiment as described previously (Nii *et al.* 2011). The extracted total RNA was dissolved in TE buffer (0.01 M Tris–HCl, pH 8.0, and 1 mM EDTA). Samples were treated with RQ1 RNase-free DNase (Promega Co.) in a 10 μl reaction mixture (0.5 μg of total RNA, $1 \times$ DNase buffer, and 1 U DNase) on a PTC-100 programmable thermal controller (MJ Research, Inc., Waltham, MA, USA), 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). RNA samples were reverse transcribed using ReverTra

Ace (Toyobo Co. Ltd., Osaka, Japan) according to the instructions of the manufacturer. The reaction mixture (10 μ l) consisted of 0.5 μ g of total RNA, 1 $\times$ RT buffer, 1 mM dNTP mixture, 20 U RNase inhibitor, 0.5 μ g oligo(dT) 20 primer, and 50 U ReverTra Ace. The RT 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.

PCR to analyze the expression of *AvBDs*, *TLR4* and *TLR21*, and *IL1R1* and *IL6R* was performed using Takara Ex Taq (Takara Bio, Inc., Shiga, Japan) according to the protocol of the manufacturer on a PTC-100 Programmable Thermal Controller. The primers for target genes and ribosomal protein S17 (*RPS17*) used in this study are shown in Table 1. The PCR mixture (25 μ l) contained a 0.5 μ l aliquot of cDNA, 1 $\times$ PCR buffer, 1.5 mM MgCl₂, 0.2 mM each dNTP, 1.25 U Takara Ex Taq, and 0.5 μ M each primer. The cycle parameters were denatured at 94 °C for 30 s, 30 cycles (for *IL1R1* and *RPS17*), or 40 cycles (*AvBD1*–*14*, *TLR4*, *-21*, and *IL6R*); annealing at 56 °C (for *AvBD3*,

AvBD5–*7*, *AvBD10*, and *AvBD14*), 58 °C (*AvBD1*, *-2*, *-8*, *-9*, *-11*, *-12*, *TLR4*, *-21*, and *IL1R1*), or 60 °C (*AvBD4*, *-13*, *IL6R*, and *RPS17*) for 30 s, and extension at 72 °C for 1 min followed by final extension at 72 °C for 6 min. The PCR products were separated by electrophoresis on a 2% (w/v) agarose gel containing 0.4% (w/v) ethidium bromide. Analysis was performed in duplicate using different cultured cell samples.

Quantitative real-time PCR

Real-time PCR was performed using the Roche Light Cycler system (Roche Applied Science) as described in our previous study (Abdelsalam *et al.* 2012). The reaction mixture (20 μ l) consisting of 1 μ l cDNA, 1 $\times$ SYBR Premix EX Taq (Takara Bio, Inc.), and 0.5 μ M each primer was placed into 20 μ l capillaries (Roche Diagnostics GmbH). The thermal protocols for PCR were at 95 °C for 5 s; 60 °C for 20 s (*AvBD10* and *RPS17*), 60 °C for 30 s (*AvBD1* and *AvBD4*), or 63 °C for 30 s (*AvBD3*, *-5*, and

Table 1 Primer sequences for *AvBDs*, *IL1B*, *IL6*, *TLRs*, *IL1R1*, *IL6R*, and *RPS17*.

Target genes	Sequences 5'–3'	Accession no.	References
<i>AvBD1</i>	F: AAACCATGCGGATCGTGACCTGC R: CAATGCTAAACTGCACACCTTTA	AF033335	Subedi <i>et al.</i> (2007)
<i>AvBD2</i>	F: GTTCTGTAAAGGAGGGTCTGCCAC R: ACTCTACAACACAAAACATATTGC	AF033336	Subedi <i>et al.</i> (2007)
<i>AvBD3</i>	F: CTGCCGCTTCCACACATAG R: GCAATGCCAACTGCACGCCTTTA	NM_204650	Subedi <i>et al.</i> (2007)
<i>AvBD4</i>	F: ATCGTGCTCCTCTTTGTGGCAGTTCA R: CTACAACCATCTACAGCAAGAATACT	NM_001001610	Subedi <i>et al.</i> (2007)
<i>AvBD5</i>	F: ATGCAGATCCTGCCTGCCTCTCTTTGCT R: TCAGGAATACCATCGGCTCCGGCAGCAGAA	NM_001001608	Subedi <i>et al.</i> (2007)
<i>AvBD6</i>	F: GATCCTTTACCTGCTGCTGCT R: TCCTCACACAGCAAGATTTAGTC	NM_00100193	Subedi <i>et al.</i> (2007)
<i>AvBD7</i>	F: CTGCTGCTGTCTCTTTGTGG R: CATTGGTAGATGCAGGAAGGA	NM_001001194	Subedi <i>et al.</i> (2007)
<i>AvBD8</i>	F: TTCTCCTCACTGTGCTCCAA R: AAGGCTCTGGTATGGAGGTG	NM_001001781	Watanabe <i>et al.</i> (2011)
<i>AvBD9</i>	F: ATGAGAATCCTTTTCTTCTTGTTC R: TTAGGAGCTGGGTGCCCATTTGCAGC	NM_001001611	Subedi <i>et al.</i> (2007)
<i>AvBD10</i>	F: CTGTTCTCCTCTTCTCTTCCAG R: AATCTTGGCACAGCAGTTTAACA	NM_001001609	Subedi <i>et al.</i> (2007)
<i>AvBD11</i>	F: ACTGCATCCGTCCAAAGCTCG R: GTCCAGCTGTTCTTCCAG	NM_001001779	Ebers <i>et al.</i> (2009)
<i>AvBD12</i>	F: GGAACCTTTGTTTCGTGTTCA R: GAGAATGACGGGTCAAAGC	AY534898	Abdelsalam <i>et al.</i> (2012)
<i>AvBD13</i>	F: CATCGTTGTCAATTCTCTCTCTC R: ACTTGACGCTGTGGGAGTTG	NM_001001780	Subedi <i>et al.</i> (2007)
<i>AvBD14</i>	F: CATATTCCTCTGTTTCTTGTCTC R: GCCAGTCCATTGTAGCAGGT	AM402954	Watanabe <i>et al.</i> (2011)
<i>TLR4</i>	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	AY064697	Zhang <i>et al.</i> (2012)
<i>TLR21</i>	F: TGCCCCCTCCCACTGCTGTCCACT R: AAAGGTGCCTTGACATCCT	NM001030558	Zhang <i>et al.</i> (2012)
<i>IL1B</i>	F: GGGCATCAAGGGCTACAA R: CTGTCCAGGCGGTAGAAGAT	NM_204524	Nii <i>et al.</i> (2011)
<i>IL6</i>	F: AGAAATCCCTCCTCGCCAAT R: AAATAGCGAACGGCCCTCA	NM_204628.1	Nii <i>et al.</i> (2011)
<i>IL1R1</i>	F: TTGTTCACTGCTGAAGAATGTGTTATTTG R: ACGAATGTTCTGAAGTGGGTGTTT	NM_205485	*
<i>IL6R</i>	F: TGAGGATGATCCCTACGGCTATG R: CCGGCATCATCAGCAGTGT	NM_001044675	*
<i>RPS17</i>	F: AAGCTGCAGGAGGAGGAGAGG R: GGTGGACAGGCTGCCGAAGT	NM_204217	Nii <i>et al.</i> (2011)

F, forward; R, reverse. *PCR products were sequenced for verification.

-12) and then 72 °C for 1 min (AvBD1, AvBD3–5, and AvBD12). Real-time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method to calculate the relative level of mRNA in each sample and are expressed as ratios in relation to the *RPS17* housekeeping gene (Livak & Schmittgen 2001). The RNA samples obtained from unstimulated cells were used as standards.

Statistical analysis

The relative expression of *IL1B*, *IL6*, and *AvBD1*, *AvBD3–5*, *AvBD10*, and *AvBD12* is expressed as the mean $\pm$ s.e.m. ($n=6$). The significance of differences in the relative expression among different dose groups within LPS, CpG-ODN, *IL1B*, or *IL6* treatments was examined by one-way ANOVA followed by Tukey's test or the Kruskal–Wallis test. Differences were considered significant at $P<0.05$.

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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