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Human oviductal epithelial cells express Toll-like receptor 3 and respond to double-stranded RNA: Fallopian tube-specific mucosal immunity against viral infection

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BACKGROUND: The aim of this study was to evaluate the site-specific immunoregulatory mechanisms against viral infection in human Fallopian tubes. METHODS: We therefore investigated the effects of double-stranded RNA (dsRNA) on the production of interleukin (IL)-6, IL-8 and granulocyte chemotactic protein-2 (GCP-2) by cultured oviductal epithelial cells (OECs) using enzyme-linked immunosorbent assays. Phosphorylation of inhibitor κB-α (IκB-α) protein after dsRNA stimulation and the expression of Toll-like receptor (TLR) 3 in these cells were also evaluated by western blot analysis. RESULTS: Polyriboinosinic:polyribocytidylic acid (poly I:C), a synthetic dsRNA that antagonizes TLR3, stimulated the secretion of IL-6, IL-8 and GCP-2 by OECs. Poly I:C-induced production of these cytokines by OECs was inhibited by the pretreatment of these cells with anti-TLR3 antibody. The phosphorylation of IκB-α protein was detected in OECs after stimulation by poly I:C. The expression of TLR3 was also detected in OECs. CONCLUSION: These results suggest that the epithelial cells of the human Fallopian tube have evolved a unique, site-specific mechanism for recognizing viral infection. TLR3-mediated production of proinflammatory cytokines and chemokines in OECs in response to viral dsRNA may be important for antiviral immunity in the human female reproductive tract.

Key words: viral infection/Fallopian tube/double-stranded RNA/Toll-like receptor 3/nuclear factor-κB

Introduction

The mucosal surface of the female genital tract is a complex biosystem that provides a physical barrier against the outside world and participates in both innate and acquired immune defence. This mucosal compartment has adapted to a dynamic, non-sterile environment challenged by various antigenic/inflammatory stimuli associated with sexual intercourse and endogenous vaginal microbiota. Thus, the epithelial cells, fibroblasts, lymphocytes, macrophages and dendritic cells associated with the genital tract have unique features that enable them to adapt to this dynamic milieu. Despite the important role that the genital mucosa plays in immune defence, little is known about the mechanism of epithelial cell activation by pathogens or about the receptors and secondary mediators involved in this regulated response. It has been suggested that there is an oviduct-specific mucosal immune system that differs from those described for the gastrointestinal and respiratory tracts, as well as from that of the lower segments of the female reproductive tract (Cardenas *et al*., 1998). This putative oviduct-specific immune system might contribute to maintaining an aseptic milieu free of the micro-organisms that sporadically colonize the upper reproductive tract (Heinonen *et al*., 1985).

Rapid innate immune defences against microbial infection usually involve the recognition of invading pathogens by specific pattern recognition receptors recently attributed to the family of Toll-like receptors (TLRs). TLRs recognize conserved pathogen-associated molecular patterns (PAMPs) synthesized by micro-organisms but not by the host. Members of the TLR family, which includes 11 TLRs identified thus far, recognize distinct PAMP produced by various bacterial, fungal and viral pathogens. The recognition of bacterial PAMPs, such as lipopolysaccharide (LPS), peptidoglycan and flagellin, is mediated by TLR1, 2, 4, 5 and 6 (Lien *et al*., 1999; Takeuchi *et al*., 1999; Hayashi *et al*., 2001; Kirschning and Schumann, 2002). Among these TLRs, four are designed to recognize nucleic acids, namely TLR3, TLR7, TLR8 and TLR9 (Hemmi *et al*., 2000; Alexopoulou *et al*., 2001; Diebold *et al*., 2004; Heil *et al*., 2004). TLR7 and TLR8 recognize nucleotide derivatives, such as self and viral single-stranded RNA (ssRNA) (Diebold *et al*., 2004; Heil *et al*., 2004), and TLR9 binds unmethylated DNA found in bacteria (Hemmi *et al*., 2000). By contrast, TLR3 recognizes double-stranded RNA (dsRNA) (Alexopoulou *et al*., 2001), a molecular signature of RNA viruses (Bowie and Haga, 2005). Therefore, it is likely that

TLR3 plays a physiological role in antiviral innate immunity (Matsumoto *et al*., 2002).

DsRNA is generated during RNA viral infection and induces antiviral responses in host cells through a TLR3-mediated signalling pathway. Polyriboinosinic:polyribocytidylic acid (poly I:C) is a synthetic dsRNA that binds TLR3 and elicits various cellular responses similar to those provoked by viral infection (Alexopoulou *et al*., 2001; Matsumoto *et al*., 2002). The induction of TLR3 signalling via dsRNA activates transcription factors such as nuclear factor (NF)-κB and interferon regulatory factor 3 (IRF3), resulting in the production of proinflammatory and antiviral cytokines (Alexopoulou *et al*., 2001; Taniguchi *et al*., 2001; Doyle *et al*., 2002; Sato *et al*., 2003).

TLR3 is widely expressed in cells of the innate immune system, such as macrophages and dendritic cells, as well as in non-immune cells. In the female reproductive tract, TLR3 expression has been demonstrated in the epithelial cells of the vagina (Fazeli *et al*., 2005), uterine cervix (Fichorova *et al*., 2002; Fazeli *et al*., 2005), endometrium (Fazeli *et al*., 2005; Jorgenson *et al*., 2005; Schaefer *et al*., 2005) and Fallopian tubes (Fazeli *et al*., 2005). Poly I:C has been reported to induce the expression of proinflammatory cytokines [e.g. tumour necrosis factor (TNF)-α and interleukin (IL)-6] and chemokines [e.g. IL-8, interferon-γ-inducible protein-10 (IP-10), regulated upon activation, normal T cell expressed and secreted (RANTES) and monocyte chemoattractant protein-1 (MCP-1)] by endometrial epithelial cells (Jorgenson *et al*., 2005; Schaefer *et al*., 2005) via TLR3-mediated signalling pathways. However, little is known about the role of viral dsRNA in human Fallopian tubes.

In this study, we investigated (i) the expression of TLR3, (ii) dsRNA-induced activation of NF-κB and (iii) dsRNA-stimulated production of proinflammatory cytokines and chemokines in cultured human oviductal epithelial cells (OECs). The role played by TLR3-mediated signalling in the recognition of viral dsRNA in the mucosal immune defence of the human Fallopian tube is also addressed.

Materials and methods

Human OEC isolation procedure and cell culture conditions

Normal oviducts were obtained from premenopausal patients (aged 31–40 years, $n = 12$) who had undergone sterilization or hysterectomy for leiomyoma and who showed no evidence of sexually transmitted disease or pelvic inflammatory disease with routine blood biochemical analysis, gynaecological examination and standard tests for syphilis, gonorrhoea, chlamydia, hepatitis B, hepatitis C, herpes genitalis, condyloma acuminatum and acquired immunodeficiency syndrome. All patients were in the proliferative phase and had been free of all hormonal treatments before the operation. All of the specimens were diagnosed as normal according to standard histological examination. This study was approved by the institutional review board (IRB) of the Faculty of Medicine at Oita University, and written informed consent was obtained from all patients. OECs were isolated as previously described (Itoh *et al*., 2006a,b). Briefly, the resected oviducts were immediately placed in ice-cold phosphate-buffered saline (PBS) containing 100 IU/ml of penicillin (Gibco-BRL, Gaithersburg, MD, USA) and 50 mg/ml of streptomycin (Gibco-BRL). The ampulla segments of the oviducts were separated from the connecting tissue, washed several times with PBS and placed under sterile conditions in Petri dishes containing PBS. The segments were then cut open longitudinally, and the inner lining of the lumen was removed microsurgically, cut into small pieces and suspended in PBS. The cells were washed three times with 10 ml of PBS by centrifugation. The final pellets of the inner lining containing OECs were suspended in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 100 IU/ml of penicillin (Gibco-BRL), 50 mg/ml of streptomycin (Gibco-BRL) and 10% heat-inactivated fetal bovine serum (Gibco-BRL), and the samples were then cultured in 35-mm culture dishes (Corning, NY, USA) at 37°C in 5% CO_2 in air.

Immunocytochemical staining with antibodies to vimentin (V9; Dako, Copenhagen, Denmark), cytokeratin (Dako), factor VIII (Dako) and leukocyte common antigen (2B11+PD7/26, Dako) was used to evaluate OECs in a monolayer culture after the first passage, and these OECs were used for the following experiments. Each experiment was performed in triplicate and repeated at least four times.

Detection by enzyme-linked immunosorbent assay of IL-6, IL-8 and granulocyte chemotactic protein-2 in culture media of OECs

To study the production of IL-6, IL-8 and granulocyte chemotactic protein-2 (GCP-2) by OECs, we plated 1×10^6 cells on 24-well culture plates (Corning) and cultured the cells until they were fully confluent. The supernatant was then replaced with 1 ml of fresh culture medium containing various amounts of poly I:C (1–100 μg/ml) (Amersham Biosciences, Piscataway, NJ, USA) and poly dI:dC (1–100 μg/ml) (Amersham Biosciences). Under these conditions, the supernatant was collected 24 h after stimulation and stored at –70°C until assay. The time at which stimulation was performed was determined by a time course study performed as a background experiment. The cells isolated from each individual patient were used for individual experiments, and each experiment was performed in triplicate. All experiments were performed in the presence of 10% heat-inactivated fetal bovine serum. The concentrations of IL-6, IL-8 and GCP-2 were determined in each supernatant with commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). The sensitivities of the assay were 0.70 pg/ml for IL-6, 4.4 pg/ml for IL-8 and 1.6 pg/ml for GCP-2.

For antibody-blocking experiments, OECs were pretreated with anti-TLR3 monoclonal antibody (TLR3.7) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or an IgG1 isotype control (Dako) at a final concentration of 20 μg/ml for 1 h at 37°C, then stimulated with poly I:C for 24 h.

Western blot analysis

The OECs were plated in 10-cm dishes and further cultured until confluence. Then poly I:C (100 μg/ml) and poly dI:dC (100 μg/ml) were added to the cells, which were further cultured for 5 min. The time at which stimulation was performed was determined by a time course study performed as a background experiment. After stimulation, the cells were washed with PBS, and whole-cell extracts were prepared by lysing the cells in lysis buffer (50 mM Tris–HCl, 125 mM NaCl, 0.1% Nonidet P-40, 5 mM ethylenediamine tetraacetic acid, 50 mM NaF and 0.1% phenylmethylsulphonylfluoride). The suspension was centrifuged at 15 000 g for 15 min at 4°C, and the supernatant was collected. The total protein concentration was quantified using Coomassie protein assay reagent (Pierce, Rockford, IL, USA). The whole-cell protein extract was resolved with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) using 10% polyacrylamide gel under reduced conditions. After transfer of each sample to an Immobilon-P transfer membrane (Millipore, Bedford, MA, USA), the protein was stained with Ponceau S (Sigma-Aldrich, St Louis, MI, USA) to verify uniform loading and transfer. The membranes were

blocked with 5% skim milk (Becton-Dickinson, Sunnyvale, CA, USA) in Tris-buffered saline with Tween-20 (50 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.4) (TBS-T) overnight, and they were subsequently incubated with primary antibodies [TLR3, phosphorylated IκB-α (pIκB-α), non-pIκB-α (Cell Signaling, Beverly, MA, USA) and glyceraldehyde-3-phosphatedehydrogenase (GAPDH) (Ambion, Austin, TX, USA)] at the appropriate dilution for 1 h at room temperature. The membranes were then washed three times with TBS-T and incubated with the appropriate horse-radish peroxidase-conjugated secondary antibody for 1 h at room temperature. Subsequently, the membranes were washed three times with TBS-T and analysed by enhanced chemiluminescence (Amersham Biosciences).

Statistical analysis

The data were presented as the means ± SD and were then analysed by the Bonferroni/Dunn test with StatView 4.5 (Abacus Concepts, Berkeley, CA, USA). $P < 0.05$ was accepted as statistically significant.

Results

The levels of IL-6, IL-8 and GCP-2 in the culture media without cells were below the levels of detection (<0.70, <4.4 and <1.6 pg/ml, respectively). Significant quantities of IL-6, IL-8 and GCP-2 protein were detected in the culture medium of non-stimulated OECs (15.4 ± 1.0, 10 040 ± 440 and 622 ± 55 pg/ml, respectively) incubated for 24 h (Figure 1). As shown in Figure 1, the levels of these cytokines in the supernatant of the OECs increased with increasing amounts of poly I:C (128 ± 21 pg/ml for IL-6, 17 800 ± 1100 pg/ml for IL-8 and 2010 ± 140 pg/ml for GCP-2 at a concentration of 100 μg/ml, respectively; $P < 0.0005$). However, poly dI:dC did not influence the concentrations of these three cytokines in the supernatant of the OECs (Figure 1).

In order to determine the involvement of TLR3 in these responses, inhibition experiments with an anti-TLR3 monoclonal antibody were performed. Preincubation of OECs with an anti-TLR3 antibody significantly inhibited the poly I:C-induced secretion of IL-6 (36.3% inhibition), IL-8 (23.1% inhibition) and GCP-2 (28.6% inhibition) relative to that seen with an IgG isotype control (Figure 2).

To analyse the underlying mechanisms for the above findings, we further evaluated the respective signal pathways of poly I:C-stimulated production of these three cytokines in the OECs. As shown in Figure 3, the non-stimulated OECs revealed significant non-pIκB-α and slight pIκB-α protein expression. A significant increase in the pIκB-α protein was observed in OECs after stimulation by poly I:C (100 μg/ml), whereas no phosphorylation of the IκB-α protein was detected after stimulation by poly dI:dC (100 μg/ml) in these cells. GAPDH was detected in all samples in almost equal quantities.

As shown in Figure 4, significant expression of TLR3 protein was also detected in the OECs.

Discussion

DsRNA during viral infection can arise from several sources (Sen and Sarkar, 2005). The genome of the infecting virion itself can be dsRNA, as in the case with the known natural

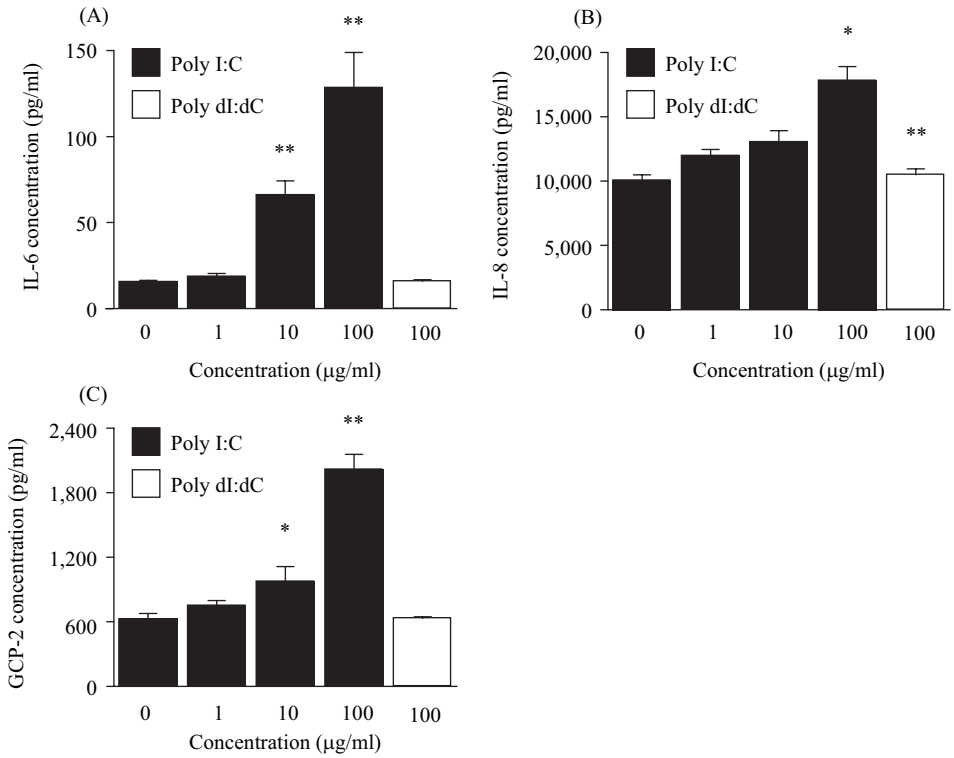

Figure 1. Levels of (**A**) IL-6, (**B**) IL-8 and (**C**) GCP-2 in the culture media of OECs after 24-h stimulation with poly I:C (closed bars) or poly dI:dC (open bars). *$P < 0.005$, **$P < 0.0005$ versus untreated controls. GCP-2, granulocyte chemotactic protein-2; IL, interleukin; poly I:C, polyriboinosinic:polyribocytidylic acid; OECs, oviductal epithelial cells.

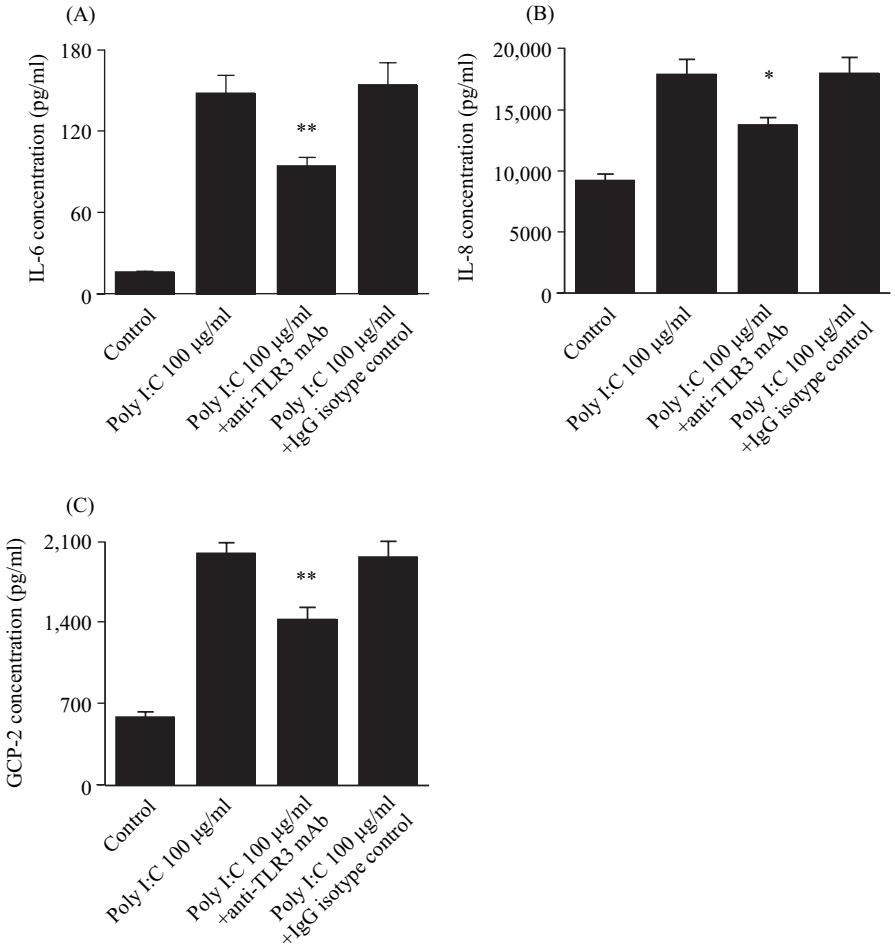

Figure 2. Effects of preincubation with anti-TLR3 antibody or IgG isotype control on the poly I:C-induced secretion of IL-6, IL-8 and GCP-2 by OECs. *$P < 0.0025$, **$P < 0.0005$ versus poly I:C-treated groups. GCP-2, granulocyte chemotactic protein-2; IL, interleukin; poly I:C, polyriboinosinic:polyribocytidylic acid; OECs, oviductal epithelial cells; TLR, Toll-like receptor.

dsRNA viruses. However, even ssRNA virus samples often contain defective particles that have packaged primarily double-stranded defective genomes. Intracellular viral dsRNA can be generated in various ways. In the case of ssRNA viruses, the formation of dsRNA replication intermediates is an obligatory step in viral reproduction. In the case of DNA viruses, complementary mRNAs are often produced that are encoded by partially overlapping genes located on the opposite strands of the viral genome. Long viral polycistronic mRNAs often contain many stable double-stranded stems. Such findings, taken together, have indicated that all viral infections induce dsRNA at some point in their replication (Jacobs and Langland, 1996). Therefore, this feature of these viral dsRNAs can be mimicked by the synthetic dsRNA, poly I:C (Sobel *et al*., 1994; Der *et al*., 1997).

TLR3 is activated by dsRNA and plays an important role in the innate immunity directed against dsRNA viral pathogens by coupling to Toll/IL-1 receptor domain-containing adapter protein-dependent signal transduction pathways (Alexopoulou *et al*., 2001; Yamamoto *et al*., 2002). TLR3 can mediate responses to the above-mentioned synthetic analogue of viral dsRNA, poly I:C, which has been used extensively in experimental studies to mimic viral infection (Alexopoulou *et al*., 2001). It has been assumed that TLR3 plays a key role in antiviral immunity against various viruses (Wang *et al*., 2004). Recently, host-derived mRNA released by dying or dead cells has been shown to activate TLR3, suggesting that activation via TLR3 can occur in various situations (Kariko *et al*., 2004). In the former case, it was demonstrated that a secondary structure creating hairpin loops within the mRNA was responsible for TLR3 activation. In addition, TLR3 has been shown to recognize double-stranded nucleic acid from *Schistosoma mansoni* and to be involved in the antiparasite response (Aksoy *et al*., 2005). At present, it is considered that RNA from a number of different sources can activate TLR3, as long as it displays a secondary structure that includes double-stranded regions and as long as it is present in the appropriate cellular vesicle.

Recently, Fazeli *et al*. (2005) demonstrated the presence of TLR3 protein in OECs, which is consistent with our findings. However, they only evaluated the distribution of TLR3 protein in the human Fallopian tubes by immunohistochemistry. In the present study, we demonstrated that cultured OECs respond to viral dsRNA by secreting proinflammatory cytokines and cysteine-X-cysteine (CXC) chemokines. This

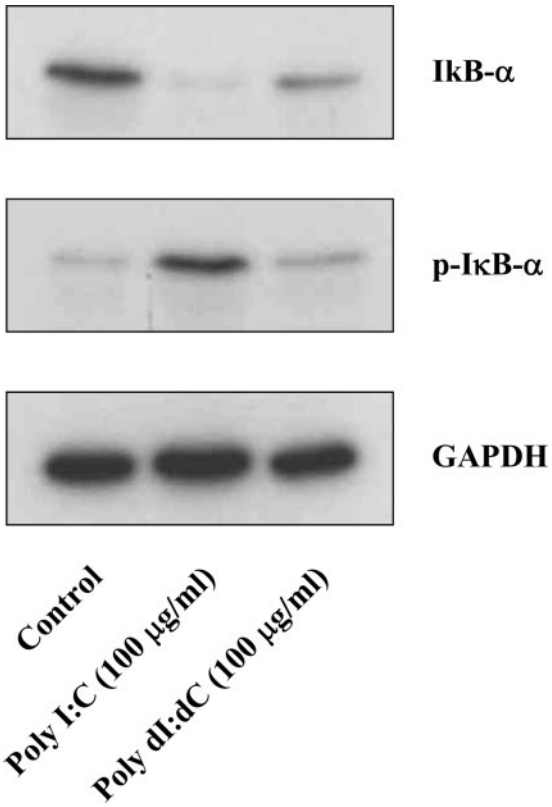

Figure 3. Effects of poly I:C (100 μg/ml) or poly dI:dC (100 μg/ml) stimulation on the levels of non-pIκB-α, pIκB-α and GAPDH proteins expressed by OECs. Poly I:C, polyriboinosinic:polyribocytidylic acid; OECs, oviductal epithelial cells.

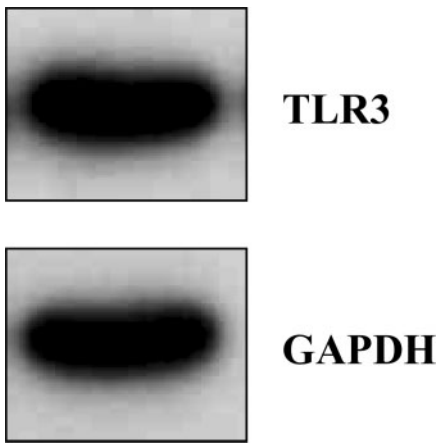

Figure 4. Expression of TLR3 and GAPDH proteins in non-stimulated OECs. OECs, oviductal epithelial cells; TLR, Toll-like receptor.

response appears to be via TLR3 and may involve the phosphorylation of IκB.

Induction of TLR3-mediated signalling via dsRNA activates transcription factors such as NF-κB and IRF3, resulting in the production of proinflammatory cytokines and chemokines, as well as antiviral cytokines, which are necessary for subsequent immune responses (Alexopoulou *et al*., 2001; Taniguchi *et al*., 2001; Doyle *et al*., 2002; Sato *et al*., 2003). In the light of these findings, we examined the effects of poly I:C on the production of IL-6 and CXC chemokines and the activation of NF-κB and the presence of TLR3 in OECs. Our present findings demonstrated that TLR3 protein is expressed in OECs and that stimulation of OECs with dsRNA leads to the phosphorylation of IκB, as well as to the secretion of IL-6, IL-8 and GCP-2, presumably through TLR3. The IL-6 and CXC chemokines are key mediators that orchestrate host immunoinflammatory responses to viral infections. Those that activate leukocytes, particularly polymorphonuclear neutrophils and T lymphocytes, are essential for the early, non-specific eradication of invading microbes. It is essential that the normal oviductal epithelium has the capacity to recognize and immediately respond to viral infections. In the clinical situation, various viruses including herpes simplex virus, human papilloma virus, hepatitis B virus, hepatitis C virus, cytomegalovirus and human immunodeficiency virus may become the causative of urogenital viral infection.

The mucosal surfaces of the corneal, respiratory and gastrointestinal tracts, as well as those of the urogenital tracts, separate the external environment from the internal sterile environment, thereby representing the first line of defence against microbes. These surfaces are not just a simple physical barrier but also possess chemical and cellular defences, which differ slightly from one tract to another, because of the different internal pressures these systems have to face (Basset *et al*., 2003). TLR3 expression has been demonstrated in the epithelial cells of various tissues including the skin (Tohyama *et al*., 2005), cornea (Ueta *et al*., 2005), nasal mucosa (Fransson *et al*., 2005), airway (Sha *et al*., 2004; Guillot *et al*., 2005) and intestine (Furrie *et al*., 2005). These epithelial cells produce various proinflammatory cytokines, chemokines and antiviral cytokines in response to poly I:C stimulation. To the best of our knowledge, the absence of TLR3 expression in epithelial cells has not been reported in the luminal organs of human beings. It is suggested that universal expression of TLR3 in the surface epithelium may be important in antiviral immunity.

Interestingly, we previously demonstrated that OECs lack TLR4 expression, and therefore, these cells do not respond to LPS stimulation (Itoh *et al*., 2006a). However, in the present study, we demonstrated that OECs express TLR3 and respond to viral dsRNA. It is suggested from these findings that the human oviductal mucosa may have distinct immune systems against viral and bacterial infection.

In conclusion, we demonstrated that viral dsRNA, a ligand for TLR3, stimulates the production of IL-6 and CXC chemokines in OECs and induces the phosphorylation of IκB. Although other receptor and signal pathways may exist for the recognition of viral infection, our present findings suggest that TLR3-NF-κB-mediated pathway is involved in the immune responses of OECs after exposure to viral dsRNA. Further investigations on the immunoregulatory mechanisms against viral infection in the human Fallopian tube may clarify the mucosal immune pathophysiology in the female genital tract. In addition, immunogenetic studies on TLR3 and other candidate genes of viral infection may provide insight

into the late complication of the Fallopian tubes after urogenital infection.

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