Hyaluronan fragments generated by sperm-secreted hyaluronidase stimulate cytokine/chemokine production via the TLR2 and TLR4 pathway in cumulus cells of ovulated COCs, which may enhance fertilization

Masayuki Shimada1*, Yoshiari Yanai1, Tetsuji Okazaki1, Noritaka Noma1, Ikkou Kawashima1, Takahide Mori2 and JoAnne S. Richards3

The toll-like receptor (TLR) system is expressed in cumulus cells of ovulated cumulus-oocyte complexes (COCs) and is activated by bacterial lipopolysaccharides (LPS). However, the endogenous ligand(s) for the TLRs and the physiological role(s) in ovulated COCs remain to be defined. Based on reports that hyaluronan fragments can activate TLR2 and TLR4 in macrophages, and that ovulated COCs are characterized by a hyaluronan-rich matrix, we cultured ovulated mouse COCs with purified hyaluronan fragments, treated them with purified hyaluronidase or exposed them to sperm as a physiologically relevant source of hyaluronidase. Hyaluronan fragments or hyaluronidase activated the NFκB pathway and induced Il6, Cc14 and Cc15 mRNA expression within 2 hours. Anti-TLR2 and anti-TLR4 neutralizing antibodies significantly suppressed hyaluronan fragment- and hyaluronidase-induced activation of the NFκB pathway and the expression of these genes. When ovulated COCs were cultured with sperm, the expression and secretion of cytokine/chemokine family members were induced in a time-dependent manner that could be blocked by TLR2/TLR4 antibodies or by a hyaluronan-blocking peptide (Pep-1). The chemokines secreted from TLR2/TLR4-stimulated COCs activated cognate chemokine receptors (CCRs) localized on sperm and induced sperm protein tyrosine phosphorylation, which was used as an index of capacitation. Significantly, in vitro fertilization of COC-enclosed oocytes was reduced by the TLR2/TLR4 neutralizing antibodies or by Pep-1. From these results, we propose that TLR2 and TLR4 present on cumulus cells were activated by the co-culture with sperm in a hyaluronan fragment-dependent manner, and that chemokines secreted from COCs induced sperm capacitation and enhanced fertilization, providing evidence for a regulatory loop between sperm and COCs during fertilization.

KEY WORDS: Toll-like receptor, Cumulus cell, Cytokine/chemokine, Fertilization, Sperm

INTRODUCTION

Ovulation is essential for successful reproduction. In mammals, this process involves the release of a mature oocyte and its surrounding cumulus cells, the cumulus cell oocyte complex (COC). The extrusion of COCs is dependent on the formation and stabilization of a matrix composed of a polymeric hyaluronan (HA) backbone and HA-binding proteins/proteoglycans (Salustri et al., 1999; Richards et al., 2002; Richards, 2005). Some of the matrix-associated genes that have been identified encode hyaluronan synthase 2 (HAS2) (Fülöp et al., 1997a), TNF-α-induced protein 6 (TNFAIP6, also known as TSG6) (Fülöp et al., 1997b; Fülöp et al., 2003) and pentraxin 3 (PTX3) (Varani et al., 2002; Salustri et al., 2004). Polymeric HA directly binds to serum-derived inter-α-trypsin inhibitor (Iten) (Chen et al., 1992; Camaioni et al., 1993) and interacts with TNFAIP6. PTX3 binds directly to TNFAIP6 to provide an additional link of stability to the matrix scaffold within COCs (Fülöp et al., 2003; Salustri et al., 2004).

The functional relevance of HA and HA-binding proteins has been documented by a wealth of biochemical data and physiological studies using mutant mouse models. Specifically, the level of Tnfaip6 mRNA was significantly lower in mice null for either Ptgs2 (prostaglandin-endoperoxide synthase 2, also known as COX2) or Ptgser2 (prostaglandin E receptor 2, also known as EP2) than in their wild-type littermates (Ochsner et al., 2003a; Ochsner et al., 2003b), indicating that Tnfaip6 gene expression was dependent on prostaglandin E2 and its receptor pathway. Ovulation in both mouse models was reduced slightly, but cumulus expansion, as well as fertilization, was completely suppressed in these mutant mice (Lim et al., 1997; Kennedy et al., 1999; Hizaki et al., 1999). Additionally, Ptgs2-null mice showed abnormal cumulus-oocyte complex morphology and reduced in vivo fertilization (Varani et al., 2002; Salustri et al., 2004). The fertilization defects of Ptgser2- or Ptgser2- Ptgs2-null mice were related to defective matrix stability/function because mature oocytes retrieved from these mutant mice could be fertilized in vitro by the use of capacitated spermatozoa (Matsumoto et al., 2001; Salustri et al., 2004). Collectively, these results suggest that the hyaluronan-rich matrix produced by COCs is essential not only for ovulation, but also for in vivo fertilization, and perhaps more specifically for sperm capacitation.

Specific biochemical roles of the HA-rich matrix have been implicated by recent studies showing that HA fragments can impact immune cell responses by binding to specific membrane receptors. CD44, a cell surface molecule that is generally considered to be a primary HA receptor (Aruffo et al., 1990; Miyake et al., 1990), is expressed in cumulus cells of mouse, human and porcine COCs during the ovulation process (Ohta et al., 1999; Kimura et al., 2002; Hernandez-Gonzalez et al., 2006). CD44 recognizes both high molecular weight HA present within extracellular matrices and small
fragments of HA generated during tissue injury, inducing the expression of cytokine and chemokine families (Hodge-Dufour et al., 1997; Cuff et al., 2001; Kothapalli et al., 2007). HA fragments have also been reported to stimulate members of the pathogen recognition receptor surveillance pathway, toll-like receptor 2 (TLR2) and TLR4, which are present in macrophages (Termeer et al., 2002; Fieber et al., 2004). Interestingly, Chang et al. reported that TLR2 and TLR4 do not recognize the high molecular weight of HA, but that fragments of less than 230 kDa can activate these receptors (Chang et al., 2007); maximal effects were observed with 30-mer fragments, suggesting a gradient of responses during HA degradation. Moreover, very small HA fragments can activate the TLR-dependent pathway but not CD44 in acute lung injury (Jiang et al., 2005), providing additional evidence that HA may exert pleomorphic effects on TLR2/TLR4-positive cells. Our recent study (Shimada et al., 2006) documented that cumulus cells of ovulated COCs express numerous immune cell-related genes, including members of the TLR family, TLR4 and related molecules. When the ovulated COCs were cultured with the TLR4 ligand, LPS, the expression of Il6, Pgts2 and Tnfα mRNA was increased. These results indicated that the TLR4 pathway was present and functional in cumulus cells of ovulated COCs. Although the physiological relevance of the TLR pathway in ovulation and fertilization has not been explored, the ability of capacitated sperm to secrete hyaluronidase, leading to the modification and breakdown of the COC HA-rich matrix, is well known (Srivastava et al., 1965; Rogers and Morton, 1973; Talbot and Franklin, 1974). These reports, and our previous study, led us to hypothesize that, during the fertilization process, sperm-secreted hyaluronidase would generate small HA fragments capable of activating TLR2 and/or TLR4 on cumulus cells.

To examine this hypothesis, ovulated COCs were cultured with the TLR4 ligand LPS, the TLR2 ligand Pam3Cys-Ser-(Lys)4 HCl (Pam3Cys), or with small HA fragments. The functional responses of cumulus cells to these ligands were analyzed by determining ligand-mediated secretion of specific cytokines and chemokines. Neutralizing antibodies for TLR2, TLR4 or CD44 were used to verify receptor activation. siRNA approaches were used to examine the responses (or lack thereof) in a granulosa cell culture system. Hyaluronidase and in vitro fertilization of COC-enclosed oocytes by sperm provided further evidence consistent with a role for HA-induced TLR activation in the fertilization process. Finally, we show that TLR2 and TLR4 are expressed by the cumulus cells of ovulated human COCs, and that a positive correlation occurs between the in vitro fertilization of human oocytes and the levels of chemokine family members secreted by the COC. Therefore, the TLR pathway may have physiological relevance in human fertility as well.

**MATERIALS AND METHODS**

**Materials**

Equine chorionic gonadotropin (eCG) and human CG (hCG) were purchased from Asuka Seiyaku (Tokyo, Japan). DMEM:F12 medium and penicillin-streptomycin were from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from Life Technologies (Grand Island, NY). Oligonucleotide poly-(dT) was purchased from Amersham Pharmacia Biotech (Newark, NJ), and AMV reverse transcriptase and Taq polymerase were from Promega (Madison, WI). α-amanitin and LPS were purchased from Sigma Chemical Co. (Sigma: St Louis, MO), and Pam3Cys was from Calbiochem (Los Angeles, CA). Hyaluronan fragments purified from pig skin were obtained from Saikagaku Kougyou (Tokyo, Japan). CCL2 (MCP1), CCL4 (MIP1β) and CCL5 (RANTES) were from R&D Systems (Minneapolis, MN). Anti-TLR2 neutralizing antibody (MAb mTLR2) and anti-TLR4 neutralizing antibody (MAb mTLR4/M2D) were purchased from InvivoGen (San Diego, CA), anti-CD44 neutralizing antibody (A020) from Calbiochem, and anti-CCL5 neutralizing antibody (anti-mouse CCL5/RANTES antibody) from R&D Systems. Routine chemicals and reagents were obtained from Nakarai Chemical Co. (Osaka, Japan) or Sigma.

**Animals**

Immature female C57BL/6 mice were obtained from Clea Japan (Tokyo, Japan). At 23 days old, female mice were injected intraperitoneally (IP) with 4 IU of eCG, to stimulate follicular growth, followed 48 hours later by injection with 5 IU hCG to stimulate ovulation and luteinization (Robker et al., 2000). Animals were housed under a 16-hour light/8-hour dark schedule in the Experiment Animal Center at Hiroshima University, and provided food and water ad libitum. Animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, as approved by the Animal Care and Use Committee at Hiroshima University.

**Cumulus-oocyte complex isolation and culture**

Ovulated COCs were recovered from oviducts and 50 COCs were cultured in separate wells of a Falcon 96-well plate (Becton Dickinson, Franklin Lakes, NJ) in 150 μl of defined medium (Ochsner et al., 2003a), containing 1% (v/v) FBS with LPS, Pam3Cys or HA fragment in the presence or absence of each specific neutralizing antibody (anti-TLR2 rat polyclonal IgG, anti-TLR4 rabbit polyclonal IgG or anti-CD44 rabbit polyclonal IgG). After culture of COCs, the culture medium was recovered for use in the Bio-Plex Protein Array System (BioRad, Hercules, CA, USA). Total RNA or protein was extracted from cumulus cells isolated from COCs (see below).

**In vitro fertilization**

Ovulated COCs were collected from oviductal ampullae 16 hours after the hCG injections and placed into 150 μl of human tubal fluid (HTF) medium. Spermatozoa were collected from the cauda epididymes of 4-month-old ICR strain mice into 500 μl of HTF medium. After 15, 30 or 60 minutes, the spermatozoa were introduced into fertilization medium at a final concentration of 1000 spermatozoa/μl. Twelve hours after insemination, oocytes were washed thoroughly five times, and then checked for the formation of pronuclei under a phase-contrast microscope. Some COCs were recovered after 2 or 4 hours of culture with sperm; cumulus cells were then isolated to prepare the total RNA.

**Synthesis of Pep-1 and control peptide**

Pep-1 (GAHWQNALTVR) and scrambled control peptide (WRHGFLATNVQ), both with an amidated GGGS linker (Mummert et al., 2000; Jiang et al., 2005), were synthesized by Scrum (Tokyo Japan). Peptide solutions were prepared immediately before use by dissolution in DMSO to a concentration of 500 mg/ml. The COCs were pre-cultured with 0.5 mg/ml peptides (final concentration of DMSO was 0.1%) for 30 minutes, and then treated with hyaluronidase or cultured with sperm as described above. The scrambled control peptide did not significantly affect the gene expression in cumulus cells and sperm penetration (data not shown).

**Sperm culture**

Spermatozoa were collected from the cauda epididymes of 4-month-old mice into 500 μl of HTF medium. The sperm were cultured with 100 pg/ml of CCL2, CCL4 or CCL5 for 30 or 60 minutes. After culture, sperm were lysed by Laemmli sample buffer and then analyzed for tyrosine phosphorylation as described below.

**Sperm accumulation assay**

Sperm were collected from the cauda epididymes of 4-month-old mice into 500 μl of HTF medium and placed into an m-Slide VI flow chamber for live cell analysis (Ibidi GmbH, Munich, Germany). The chamber plate has two wells connected by a narrow channel. First, 100 μl of HTF medium was added to the plate and 1×10⁵ sperm were placed on one side. The agonists were added to other side chamber, and the plate was incubated for 30 minutes. After culture, the number of sperm that had moved to other side was counted.
siRNA treatment procedure in cultured mouse granulosa cells

TLR2 and TLR4 Silencer Pre-designed siRNA were purchased from Ambion (Austin, TX). The sequences were:

TLR2: GGCAGUAAGGUCUCGGAUUt (sense) and AUUCGGGAGCUUAAUGCtt (antisense);

TLR4: GCAUCAUGCAUAUUUGtt (sense) and ACAAAUGCAUCAUGCtt (antisense).

Scrambled siRNA duplex (Ambion) was used as a negative control. Mouse granulosa cells (1 × 10^6 cells/well) recovered from eCG/hCG-primed mice were plated in 12-well culture plates for 3 hours before transfection. Transfection of siRNA (25 nM) was accomplished using the HVJ envelope vector kit GenomONE neo (Ishihara Sangyo, Tokyo, Japan), according to the manufacturer’s instructions and our previous study (Shimada et al., 2007). Cells were incubated at 37°C in a CO₂ incubator, and the culture medium was replaced 5 hours after transfection. After transfection, granulosa cells were cultured with 100 μg/ml of HA fragment for 2 hours. After culture, total RNA was collected and gene expression analyzed by real-time PCR.

Collection of human COC from periovulatory follicles, and the media after in vitro fertilization

Women were stimulated with human menopausal gonadotrophin (HMG; HMG Injection TEIZO, Teikoku-zouki, Tokyo, Japan) according to routine procedures. Ovarian follicle diameter was assessed by transvaginal sonography, and gonadotropins were administered daily until the second largest follicle reached a diameter of 18 mm. When the follicle grew beyond 80% of the largest follicle, transvaginal puncture of each follicle was performed using an 18-gauge needle under ultrasonographic guidance. The oocytes underwent conventional IVF and embryo transfer on the day of the second (IVF-2) or third (IVF-3) day after hCG injection. Media after in vitro fertilization and embryo transfer were analyzed with the Bio-Plex Protein Array System (BioRad) during culture, media samples were collected and cytokines present in the media were analyzed.

Western blot analyses

Protein samples from cumulus cells, sperm, spleen or testes were prepared by homogenization in whole cell extract buffer and then diluted by the same volume of 2× SDS sample buffer (Hernandez-Gonzalez et al., 2006). Extracts (5 μg protein) were resolved by SDS polyacrylamide gel (10%) electrophoresis and transferred to Immobilon-P nylon membranes (Millipore, Bedford, MA). Membranes were blocked in Tris-buffered saline and Tween 20 (TBST: 10 mM Tris (pH 7.5), 150 mM NaCl and 0.05% Tween 20), containing 5% (w/v) non-fat Carnation instant milk (Nestle, Solon, OH). Blots were incubated with primary antibody as shown in Table 2 overnight at 4°C. After washing in TBST, enhanced chemiluminescence (ECL) detection was performed by using ECL western blotting detection reagents (Amersham) and appropriate exposure of the blots to Kodak X-ray film. Specific bands were quantified by densitometric analyses using a Gel-Pro analyzer (Media Cybernetics, Bethesda, MD).

Immunofluorescence

Testes were recovered from 4- to 5-month-old male mice, and were embedded in OCT compound (Tissue-TEK, Miles, Elkhart, IN) and stored at –80°C before the preparation of 5 μm sections, which were fixed overnight in PBS-buffered 4% (w/v) paraformaldehyde at 4°C. Sections were then sequentially probed with primary anti-CCR3 antibody (10 μg/ml, Anaspec, San Jose, CA) or normal rabbit IgG (10 μg/ml, Sigma) and secondary FITC-conjugated goat anti-rabbit IgG antibodies (diluted 1:250, Sigma). Slides were mounted using VectaShield with DAPI (Vector Laboratories, Burlingame, CA).

Bio-Plex protein array system

During the culture, media samples were collected and cytokines present in the media were analyzed with the Bio-Plex Protein Array System (BioRad) during culture. Media samples were prepared by bio-Plex Mouse Cytokine 9-Plex Panel (BioRad), including antibodies for IL1α, IL1β, IL2, IL4, IL5, IL6, IL9, IL10, IL12 (p70), IL17, Eotaxin (CCL11), granulocyte colony-stimulating factor (GCSF), granulocyte-macrophage colony stimulating factor (GMCSF), interferon-γ (IFNγ), keratinocyte-derived chemokine (KC), monocyte chemotactic protein 1 (MCP1, CCL2), macrophage inflammatory protein 1α (MIP1α, CCL3), MIP1β (CCL4), RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted: CCL5), and tumor necrosis factor-α (TNFα), or the Human Cytokine 9-Plex Panel, including IL1α, IL1β, IL2, IL4, IL5, IL6, IL9, IL10, IL12 (p40), IL12 (p70), IL13, IL17, Eotaxin (CCL11), GCSF, MCP1 (CCL2), MIP1β (CCL4), and RANTES (CCL5), as indicated in Table 1.
Statistics
Statistical analyses of data from three or four replicates were carried out by one-way ANOVA followed by Duncan’s multiple-range test (Statview; Abacus Concepts, Berkeley, CA).

RESULTS
TLR2 and TLR4 expressed on cumulus cells of ovulated COCs are functional, and both receptors are activated by HA fragments
To analyze the induction of TLRs and related molecules in cumulus cells of COCs during the ovulation process, COCs were isolated from ovaries of eCG-primed mice before (0 hours) and at 8 hours after hCG administration, as well as from oviducts at 16 hours post-hCG administration (Fig. 1A). Western blot analyses using antibodies to TLR2, TLR4, MYD88, IRF3, and 1xβ and NFκB showed that the levels of these proteins were increased in COCs collected at 8 and 16 hours post-hCG administration compared with at 0 hours, except for NFκB, confirming and extending previous studies (Shimada et al., 2006) that the TLR2/TLR4 pathway is expressed in cumulus cells of ovulated COCs. To determine if the TLR2/TLR4 pathways were functional, 23 different cytokines and chemokines were analyzed in the medium of COCs that had been cultured for 24 hours with the TLR4 ligand (LPS), the TLR2 ligand (Pam3Cys) or the small HA fragment, using the Bio-Plex Protein Array system.

As shown in Table 3, COCs cultured with LPS or Pam3Cys secreted increased levels of several cytokines and chemokines [IL1α, IL1β, IL6, IL9, IL12 (p40), IL12 (p70), GCSF, Eotaxin, CCL2, CCL3, CCL4 and CCL5] compared with COCs cultured without agonists. When COCs were cultured with HA fragments, the levels of IL6, as well as those of IL12 (p40), CCL2, CCL4 and CCL5, were higher than those observed in controls. Although COCs released IL1α, IL1β, I12 (p70), GCSF and CCL2, the levels between control and HA fragment-treated samples were not

Table 2. List of antibodies used for western blotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>β-Actin (AC74)</td>
<td>Sigma</td>
<td>1:10,000</td>
</tr>
<tr>
<td>CCR3</td>
<td>Anaspec, San Hose, CA</td>
<td>1:250</td>
</tr>
<tr>
<td>ERK 1/2 (p44/p42 MAP kinase, 9102)</td>
<td>Cell Signaling, Boston, MA</td>
<td>1:1000</td>
</tr>
<tr>
<td>pERK1/2 (phospho-p44/p42 MAPK, E10, 9106)</td>
<td>Cell Signaling</td>
<td>1:2000</td>
</tr>
<tr>
<td>1xβ (L35A5, 4814)</td>
<td>Cell Signaling</td>
<td>1:2000</td>
</tr>
<tr>
<td>IRF3</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>MYD88</td>
<td>eBioscience, San Diago, CA</td>
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</tr>
<tr>
<td>NF-κB (3034)</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
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<td>phospho-NF-κB p65 (Ser536, 3031)</td>
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<td>1:1000</td>
</tr>
<tr>
<td>phospho-p38MAPK (28B10, 9126)</td>
<td>Cell Signaling</td>
<td>1:2000</td>
</tr>
<tr>
<td>Toll-like receptor 2 (2229)</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>Toll-like receptor 4 (IMG578A)</td>
<td>IMGENEX, San Diago, CA</td>
<td>1:500</td>
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<tr>
<td>phospho-Tyr (P-Tyr-100, 9411)</td>
<td>Cell Signaling</td>
<td>1:5000</td>
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Fig. 1. TLR2 and TLR4 expressed on cumulus cells of ovulated COCs are functional, and both receptors can recognize HA fragments. (A) Kinetic changes in the expression of TLRs and related molecules in cumulus cells of COCs during the ovulation process. Results in each panel are representative of two separate experiments. (B) TLR2/TLR4 agonists and HA fragments induce the expression of cytokine and chemokine mRNA. Ovulated COCs were cultured with 100 ng/ml LPS, 1 μg/ml Pam3Cys or 100 μg/ml HA fragments for 2 hours. The 0 hour value was set as 1, and the data are presented as fold increase. Values are mean±s.e.m. of three replicates. Significant differences were observed as compared with COCs cultured without any agonists for 2 hours (Cont; *P<0.01; **P<0.05). (C) Effects of the anti-TLR2 or anti-TLR4 neutralizing antibody on agonist-induced expression of Il6 mRNA. Ovulated COCs were pre-cultured for 30 minutes with 50 ng/ml anti-TLR4 or anti-TLR2 neutralizing antibody, and then further cultured with LPS or Pam3Cys for 2 hours. (D) HA fragment-induced Il6 mRNA was suppressed by both anti-TLR2 and anti-TLR4 neutralizing antibodies, but not by anti-CD44 antibody. Cont, COCs cultured without any neutralizing antibodies; Free, COCs cultured without HA fragments.
The role of TLR in fertilization

Table 3. BioPlex Protein Array analysis of COC-secreted cytokine and chemokine family members (pg/ml) secreted following stimulation with LPS, Pam3Cys or HA fragments

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LPS</th>
<th>Pam3Cys</th>
<th>HA fragment</th>
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<tbody>
<tr>
<td>IL1α</td>
<td>6.3±1.5</td>
<td>47.9±5.3</td>
<td>60.9±5.0</td>
<td>7.0±5.67</td>
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<tr>
<td>IL1β</td>
<td>–</td>
<td>35.0±8.1</td>
<td>78.0±6.6</td>
<td>15.3±2.54</td>
</tr>
<tr>
<td>IL6</td>
<td>9.1±2.3</td>
<td>344.8±28.6</td>
<td>472.1±5.2</td>
<td>284.3±18.72</td>
</tr>
<tr>
<td>IL9</td>
<td>72.2±30.51</td>
<td>65.1±6.25</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IL12 (p40)</td>
<td>0.08±0.02</td>
<td>36.1±6.94</td>
<td>28.69±3.99</td>
<td>33.3±5.11</td>
</tr>
<tr>
<td>IL12 (p70)</td>
<td>52.4±10.36</td>
<td>139.18±11.56</td>
<td>196.8±21.68</td>
<td>27.87±6.33</td>
</tr>
<tr>
<td>GCSF</td>
<td>17.6±5.65</td>
<td>173.96±35.63</td>
<td>265.59±32.83</td>
<td>26.71±3.75</td>
</tr>
<tr>
<td>MCP1</td>
<td>23.5±3.89</td>
<td>468.8±55.88</td>
<td>506.21±42.68</td>
<td>255.8±20.63</td>
</tr>
<tr>
<td>MIP1α</td>
<td>5.1±1.17</td>
<td>182.78±30.42</td>
<td>404.74±25.88</td>
<td>2.85±0.06</td>
</tr>
<tr>
<td>MIP1β</td>
<td>11.0±2.20</td>
<td>1082.73±105.36</td>
<td>927.3±62.16</td>
<td>678.5±72.39</td>
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<tr>
<td>RANTES</td>
<td>24.19±5.26</td>
<td>1368.25±256.82</td>
<td>474.58±48.67</td>
<td>674.2±51.78</td>
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</tbody>
</table>

Ovulated COCs (50 COCs/well) were cultured with 100 ng/ml LPS, 1 μg/ml Pam3Cys or 100 μg/ml of HA fragments for 24 hours. Media samples were collected and cytokines present in the media were analyzed using a Bio-Plex Mouse Cytokine 23-Plex Panel.

dramatically different. The secreted levels of other tested cytokines/chemokines were below the detection limit. The dramatic increase in the secretion of specific cytokine family members in response to LPS, Pam3Cys or HA fragments confirmed the concomitant upregulation of gene expression (Fig. 1B) and previous studies stating that cumulus cells are highly secretory (Shimada et al., 2007).

To determine the mechanisms by which HA fragments stimulated gene expression in cumulus cells, we used neutralizing antibodies to TLR2, TLR4 or CD44. The anti-TLR4 neutralizing antibody significantly suppressed LPS-induced expression of Il6 mRNA but did not alter Pam3Cys-induced expression of this gene (Fig. 1C). By contrast, Pam3Cys-induced expression of Il6 mRNA was significantly suppressed by the anti-TLR2 neutralizing antibody (Fig. 1C). When ovulated COCs were cultured with HA fragments, the level of Il6 mRNA was decreased marginally by either the anti-TLR2 or anti-TLR4 neutralizing antibody, but was significantly reduced in the presence of both antibodies when compared with the control (no neutralizing antibody). The anti-CD44 neutralizing antibody did not impact Il6 mRNA expression in cumulus cells of COCs.

HA fragments induced the TLR2-, TLR4- and CD44-targeted signal transduction pathways

Previous studies have implicated p38MAPK phosphorylation, ERK1/2 phosphorylation and the activation of IκB-NFκB signaling as downstream targets of the TLR pathway (Kawai and Akira, 2005). Therefore, we investigated whether these signaling pathways were activated via TLRs in the cumulus cells of ovulated COCs cultured with HA fragments. As shown in Fig. 2, these pathways were activated by HA fragments, but the temporal pattern of MAP kinase phosphorylation was different from that of NFκB pathway. Specifically, phosphorylation of p38MAP kinase and ERK1/2 was rapidly but transiently upregulated: high levels at 15 minutes returned to basal levels after 60 minutes. By contrast, the phosphorylation of NFκB was induced progressively from 15 minutes to 120 minutes. At that time, degradation of IκB was also detected.

To determine if the regulation of MAP kinase family and NFκB pathways was mediated by TLR2/TLR4, ovulated COCs were cultured in the presence of both anti-TLR2 and anti-TLR4 neutralizing antibodies (anti-TLRs), or the anti-CD44 neutralizing antibody for 15 minutes or 2 hours, respectively. The rapid phosphorylation of p38MAP kinase and ERK1/2 by HA fragments at 15 minutes was suppressed by both anti-TLRs and anti-CD44 antibodies, suggesting that HA activated the TLR receptors. Conversely, anti-TLR2 and anti-TLR4 neutralizing antibodies suppressed the decrease of IκB and the phosphorylation of NFκB at 2 hours. Anti-CD44 antibody did not affect the NFκB pathway, at least not during the time interval examined.

Fig. 2. HA fragments induced the TLR2-, TLR4- and CD44-targeted signal transduction pathways. (A) Time-dependent changes of the activation of p38MAP kinase, ERK1/2 and the NFκB pathway in cumulus cells of ovulated COCs cultured with 100 μg/ml of HA fragments. Results in each panel are representative of two separate experiments. (B) Ovulated COCs were pre-cultured with neutralizing antibodies (both anti-TLR2 and anti-TLR4, or anti-CD44) for 30 minutes, and further cultured with HA fragments for 15 minutes (p38MAP kinase and ERK1/2), or for 2 hours (IκB and NFκB). Results in each panel are representative of two separate experiments.
Hyaluronidase impacts gene expression and NFκB pathway activation in cumulus cells of ovulated COCs

When ovulated COCs were incubated with different concentrations of hyaluronidase for 2 hours, significant increases in the expression of Il6, Ccl4 and Ccl5 mRNA were detected at the 1.0 IU/ml dose, and further increases in Ccl5 mRNA were detected at hyaluronidase concentrations of 10 or 100 IU/ml (Fig. 3A). Hyaluronidase (10 IU/ml) also significantly stimulated Ccl2 mRNA expression (Fig. 3A).

The addition of anti-TLR2 and anti-TLR4 neutralizing antibodies to the hyaluronidase-containing medium significantly decreased the expression levels of Il6, Ccl2, Ccl4 and Ccl5 mRNA in cumulus cells of COCs, as compared with those in COCs treated with 10 IU/ml hyaluronidase alone (Fig. 3B). However, Snap25 mRNA expression, which is involved in the exocytosis of these secreted factors, was not impacted by hyaluronidase treatment and by the neutralizing antibodies (Fig. 3B). Hyaluronidase treatment induced the degradation of IkBα and the phosphorylation of NFκB in cumulus cells of COCs, responses that were reversed by anti-TLR2 and anti-TLR4 neutralizing antibodies (Fig. 3B).

TLRs mediate the expression and secretion of cytokine/chemokine families during in vitro fertilization procedures

The expression of Il6 and Ccl5 mRNA in cumulus cells was also induced within 2 hours by the co-culture of ovulated COCs with sperm (Fig. 4A). The induction of these genes in cumulus cells was suppressed by the addition of anti-TLR2 plus anti-TLR4 neutralizing antibodies, or by treatment with the HA-blocking peptide Pep-1, described by Mummert et al. (Mummert et al., 2000), whereas the anti-CD44 antibody had no significant effect (Fig. 4A). These treatments did not significantly affect the expression of Snap25 mRNA (Fig. 4A). To analyze the secretion levels of cytokines/chemokines, we collected the medium 0.5, 1, 2 or 4 hours after ovulated COCs were cultured with capacitated sperm. The results show that high levels of IL6, CCL4 and CCL5 were secreted within 4 hours culture. The secretion of each cytokine was rapidly and significantly induced within 1 hour, and secretion further increased in time-dependent manner (Fig. 4B). After 1 hour of culture, the release of these cytokines from COCs was independent of de novo mRNA transcription, because addition of the transcriptional inhibitor α-amanitin (10 μg/ml) did not alter the levels of CCL4, CCL5 or IL6 in the culture medium (Fig. 4C). However, α-amanitin did significantly suppress the levels of these cytokines after 2 hours culture with sperm (Fig. 4C). Moreover, when COCs were cultured with sperm in the presence of anti-TLR2 plus anti-TLR4 neutralizing antibodies, significantly lower levels of cytokines were detected at both 1 and 2 hours culture (Fig. 4C). Lastly, pronuclear formation in oocytes was analyzed after 12 hours of in vitro fertilization with sperm. Approximately 70% of the oocytes were fertilized and contained two pronuclei (Fig. 4D), responses that were reduced by the addition of neutralizing antibodies to TLR2/TLR4 (Fig. 4D). The HA-blocking peptide Pep-1 also significantly suppressed pronuclear formation (Fig. 4D).

Sperm express chemokine receptors (CCR1, CCR2, CCR3 and CCR5) that are required for sperm capacitation during the fertilization process

Cumulus cells of cultured COCs secrete various kinds of cytokines/chemokines during the sperm-mediated fertilization process (see Table S1 in the supplementary material). Of note, IL6 and CC chemokine family members (CCL2, CCL3, CCL4 and CCL5) were predominantly produced from COCs at more than 100 pg/ml. Whereas CCL2 stimulates the CCR2 receptor, CCL3 binds mainly to CCR5, whereas the anti-CD44 antibody had no significant effect (Fig. 4A). The induction of these genes in cumulus cells was suppressed by the addition of anti-TLR2 plus anti-TLR4 neutralizing antibodies, or by treatment with the HA-blocking peptide Pep-1, described by Mummert et al. (Mummert et al., 2000), whereas the anti-CD44 antibody had no significant effect (Fig. 4A). These treatments did not significantly affect the expression of Snap25 mRNA (Fig. 4A). To analyze the secretion levels of cytokines/chemokines, we collected the medium 0.5, 1, 2 or 4 hours after ovulated COCs were cultured with capacitated sperm. The results show that high levels of IL6, CCL4 and CCL5 were secreted within 4 hours culture. The secretion of each cytokine was rapidly and significantly induced within 1 hour, and secretion further increased in time-dependent manner (Fig. 4B). After 1 hour of culture, the release of these cytokines from COCs was independent of de novo mRNA transcription, because addition of the transcriptional inhibitor α-amanitin (10 μg/ml) did not alter the levels of CCL4, CCL5 or IL6 in the culture medium (Fig. 4C). However, α-amanitin did significantly suppress the levels of these cytokines after 2 hours culture with sperm (Fig. 4C). Moreover, when COCs were cultured with sperm in the presence of anti-TLR2 plus anti-TLR4 neutralizing antibodies, significantly lower levels of cytokines were detected at both 1 and 2 hours culture (Fig. 4C). Lastly, pronuclear formation in oocytes was analyzed after 12 hours of in vitro fertilization with sperm. Approximately 70% of the oocytes were fertilized and contained two pronuclei (Fig. 4D), responses that were reduced by the addition of neutralizing antibodies to TLR2/TLR4 (Fig. 4D). The HA-blocking peptide Pep-1 also significantly suppressed pronuclear formation (Fig. 4D).

Fig. 3. The effects of hyaluronidase (HY) treatment on gene expression and NFκB pathway activation in cumulus cells of ovulated COCs. (A) Dose-dependent effects of HY on the expression of cytokine and chemokine mRNA. Ovulated COCs were treated with 0 to 100 mIU/ml of HY for 2 hours. The 0 hour value was set as 1, and the data are presented as fold increase. Values are mean±s.e.m. of three replicates. Significant differences were observed as compared with COCs cultured without HY for 2 hours (Cont; *P<0.01; **P<0.05). (B) HY-induced cytokine and chemokine mRNA expression and NFκB pathway activation were suppressed by both anti-TLR2 and anti-TLR4 neutralizing antibodies (Ab). Ovulated COCs were pre-cultured with both anti-TLR2 and anti-TLR4 antibodies for 30 minutes, then 10 mIU/ml of HY was added to the medium. The addition of neutralizing antibodies significantly suppressed Il6, Ccl2, Ccl4 and Ccl5 gene expression (*P<0.01; **P<0.05). Cont, COCs cultured without HY for 2 hours. Results of western blotting in each panel are representative of two separate experiments.
also stained positive with the rabbit IgG control antibody (Fig. 5B). Whole-mount preparations of spermatozoa provided additional evidence that CCR3 is present preferentially in the mid-piece of sperm (Fig. 5C). The immunoreactive band at about 55 kDa that corresponds to CCR3 was present in spleen used as positive control. In the testis sample, a 55 kDa band (as well as three other minor bands) was detected by the anti-CCR3 antibody. Sperm contained only one immuno-positive band of the correct size, indicating that sperm do express CCR3 (Fig. 5D).

Protein tyrosine phosphorylation in spermatozoa is thought to be crucial for the acquisition of a capacitated state and the hyper-activation of motility (Naz et al., 1991). Tyrosine phosphorylation in protein extracts from sperm exposed to CCL2, CCL4 or CCL5 for 30 and 60 minutes was analyzed by western blotting using a pan anti-Tyr phosphorylation antibody. Positive immunoreactive bands were detected at ~110 kDa, ~75 kDa, 65 kDa and 50 kDa when sperm were cultured with BSA for 60 minutes (data not shown). Although the level of phosphorylation of the band at ~110 kDa was not dramatically changed from that of the control, for each ligand, the level of phosphorylation of other bands was increased within 30 minutes culture; the highest intensity was detected in sperm cultured with CCL5 for 60 minutes (Fig. 5E).

To determine the role of CCL5 during the fertilization process, we examined whether the addition of CCL5 could overcome the inhibitory effects of TLR neutralizing antibodies on fertilization, and, conversely, whether antibodies to CCL5 could reduce fertilization. The results showed that fertilization was suppressed by anti-TLR2/TLR4 neutralizing antibodies and that the addition of CCL5 slightly, but not significantly, increased the fertilization rate (Fig. 5F). However, the anti-CCL5 neutralizing antibody significantly suppressed the percentage of oocytes that completed fertilization (Fig. 5F).

It is known that when the high concentrations of sperm recovered from the cauda epididymis are cultured for more than 60 minutes (pre-culture), capacitation is induced spontaneously (Fraser, 1977). Therefore, we examined whether the prolonged pre-culture of sperm...
that allows capacitation would overcome the negative effects of the anti-TLR2/4 neutralizing antibodies and the anti-CCL5 neutralizing antibody on sperm penetration. Without pre-culture or antibody exposure, fertilization was highly variable (control, Fig. 5G). The number of oocytes fertilized increased in controls in a duration-dependent manner, with maximal success obtained when the sperm were pre-cultured for 60 minutes (i.e. complete capacitation; Fig. 5G). The addition of neutralizing antibodies significantly suppressed the penetration of sperm that were pre-cultured for 15 or 30 minutes (Fig. 5G). However, these negative effects were not detected when sperm were pre-cultured for 60 minutes and hence were fully capacitated (Fig. 5G).

\textbf{Tlr2 and Tlr4 mRNA are expressed in human cumulus cells and cytokine/chemokine production may impact human fertility}

Human cumulus cells obtained from patients undergoing an IVF protocol expressed \textit{Tlr2} and \textit{Tlr4} mRNA (Fig. 6A). To determine the relationship between human COC-secreted cytokines/chemokines and fertilization, we compared the levels of nine different chemokine family members and the rate of oocytes fertilized. Patients were separated into two groups: the Y group, in which all of the oocytes were fertilized in vitro; and the N group, in which less than half of oocytes were fertilized. We detected IL6, CCL2, CCL4 and CCL5 in the in vitro fertilization medium. The levels of IL6 and CCL2 were almost the same between two groups; however, CCL5 was slightly higher in Y group and CCL4 was significantly elevated (Fig. 6B).

\textbf{DISCUSSION}

Our previous microarray analyses revealed that cumulus cells of ovulated COCs express a unique set of genes associated with specific immune cell-like responses. The TLRs and related factors were of particular interest because they represent part of the immune cell surveillance system that monitors changes in the external environment (Hernandez-Gonzalez et al., 2006; Shimada et al., 2006). Specifically, we have documented that, when ovulated COCs were cultured with the TLR4 ligand bacterial lipopolysaccharide
The role of TLR in fertilization

antibodies, but not the anti-CD44 antibody (see Fig. S1 in the supplementary material), blocked the effects of HA. The HA-rich matrix generated during COC expansion is presumed to be of high molecular weight, as cumulus cells express primarily HAS2 (Fülöp et al., 1997a), which is known to generate HA with a broad but extremely large size (average molecular weight of more than $2 \times 10^3$ kDa) (Itano et al., 1999). Although the functions of high molecular weight HA are not entirely clear for any cell system, it can bind and activate CD44, but not TLR2 and TLR4 in macrophages (Cuff et al., 2001; Chang et al., 2007). To determine whether HA is a physiologically relevant ligand for TLRs on cumulus cells, ovulated COCs were treated with exogenous hyaluronidase or were cultured with sperm that are known to secrete hyaluronidase. The results show that exogenous hyaluronidase significantly upregulated Il6, Ccl2, Ccl4 and Ccl5 mRNA expression, and induced activation of the NFκB pathway in cumulus cells. The induction of these genes and the activation of specific signaling pathways by hyaluronidase were suppressed by anti-TLR2/TLR4 neutralizing antibodies. Moreover, the culture with sperm induced the expression of Il6 and Ccl5 mRNA through TLR2/4-dependent, but CD44-independent, mechanisms. Additionally, we used the HA blocking peptide Pep-1, which has been shown to function effectively in vitro to inhibit the binding of HA to its receptors (Mummert et al., 2000; Taylor et al., 2004). Pep-1 significantly suppressed the expression of Il6 and Ccl5 mRNA in cumulus cells and sperm penetration in in vitro fertilization assays. It has been shown that hyaluronidase depolymerizes high molecular weight HA to 10-40 kDa end products (Sampson et al., 1992), suggesting that the small HA fragments generated by sperm-secreted hyaluronidase probably activated TLR2 and TLR4 on cumulus cells, leading to cytokine and chemokine expression via the NFκB pathway.

The high level of cytokine secretion from cumulus cells 2-4 hours after exposure to sperm was dependent on the increased transcription of specific genes, as α-amanitin blocked this effect. By contrast, the initial rapid release of IL6, CCL4 and CCL5 by the TLR2/TLR4 pathway during fertilization did not require de novo mRNA synthesis. The mechanism by which the rapid release is regulated could involve an exocytosis system because cumulus cells express components of the SNARE complex (synaptosomal-associated protein 25 (Snap25), syntaxin 1a (Stx1a) and synaptotagmin 1 (Syt1)) (Shimada et al., 2007) (M.S., unpublished). In mast cells, vesicle degranulation and the release of cytokines occurs in a TLR2- and TLR4-dependent manner (Supajatura et al., 2002), via exocytosis involving SNAP25 localized to the secretory granules (Salinas et al., 2004; Stow et al., 2006). Thus, it is possible that the SNAP25-associated exocytosis system present in cumulus cells is also activated by the small HA fragment-induced TLR pathway during the fertilization process, although the precise mechanisms remain to be resolved.

The functional role of secreted CC chemokines appears to be part of a regulatory loop between COCs and sperm, because chemokines can enhance fertilization by the activation of GPCRs (CCRs) and calcium release. We show here that multiple CCRs are expressed in mouse sperm, as well as in human sperm (Isobe et al., 2002; Muciaia et al., 2005), and that CCR3 is localized to the mid-piece of mature sperm. During capacitation, the increase of Ca$^{2+}$ is observed around the mid-piece of spermatozoa (Harper et al., 2004), and the Ca$^{2+}$-dependent pathway evokes protein tyrosine phosphorylation (Carrera et al., 1996), suggesting that secreted CC chemokine families are involved in sperm capacitation. When sperm collected from cauda epididymi were cultured with CCL2, CCL4 or CCL5, increased levels of immunoreactive phosphotyrosine were detected in extracts of the CCL5 treatment group.

Fig. 6. The expression of Tlr2 and Tlr4 mRNA and the role of cytokines/chemokines in human fertility. (A) The expression of Tlr2 and Tlr4 mRNA in cumulus cells of human COCs. The cumulus cells were recovered from COCs after in vitro fertilization. (B) The relationship between the amount of secreted CCL4 and CCL5 chemokines in vitro. The cytokines present in the media were analyzed by using the Bio-Plex Human Cytokine 9-Plex Panel. Y, all oocytes were successfully fertilized; N, more than half of the oocytes were not fertilized. P values are for Y versus N.
CCL5 treatment also increased sperm motility in a dose-dependent manner (see Fig. S2 in the supplementary material). Moreover, using COC-conditioned medium, we showed that factors secreted by COCs induced not only sperm motility but also capacitation, and that these effects were suppressed by the addition of anti-CCL5 neutralizing antibody (see Fig. S3 in the supplementary material). Furthermore, the fertilization of oocytes was suppressed significantly by either anti-TLR2/TLR4 or anti-CCL5 neutralizing antibodies if short-term pre-cultured sperm was used for insemination. However, after prolonged pre-culture (over 60 minutes), which mediates complete sperm capacitation, fertilization was not impaired by the presence of neutralizing antibodies. Based on these results, we conclude that during the fertilization process, TLR2 and TLR4 present on the cumulus cells are activated by co-culture with sperm in a hyaluronan fragment-dependent manner, leading to the secretion of CCL5 and other CC chemokine family members. These, in turn, stimulate CC receptors on sperm to enhance sperm motility and to induce sperm capacitation, thereby enhancing successful fertilization. Thus, there is a functional regulatory loop between the COCs and sperm.

Finally, we report that Tlr2 and Tlr4 mRNA are also expressed in human peri-ovulatory cumulus cells, and that human COCs secrete IL6, CCL2, CCL4 and CCL5 during in vitro fertilization. Therefore, it is likely that the TLR system in human cumulus cells plays a similar role to in mouse cells in facilitating fertilization. Importantly, human oocytes collected from COCs that produced high amounts of CCL4 or CCL5 showed higher fertilization rates than did oocytes from COCs that produced low levels of these cytokines. On the basis of these data in human and mouse COCs, the addition of CC chemokines such as CCL5 might improve IVF or intracytoplasmic sperm injection (ICSI) protocols.


