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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

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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*, *Ccl4* and *Ccl5* 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 (IαI) (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 *Ptger2* (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, *Ptx3*-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 *Ptgs2*-, *Ptger2*- or *Ptx3*-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 reports 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

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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*, *Ptgs2* 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 ooctyes 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/MD2) 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 epididymi 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 (GAHWQFNALTVR) and scrambled control peptide (WRHGFALTAVNQ), 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 epididymi 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 epididymi of 4-month-old mice into 500 μl of HTF medium and placed into a 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 HTM medium was added to the plate and 1×10^5 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.

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Table 1. List of primers employed for RT-PCR and expected product size

	Gene	Forward primer	Reverse primer	Size (bp)	Anneling temperature (°C)	Cycle
Mouse	*Ccl2*	5′-GGTCCCTGTCATGCTTCTGG-3′	5′-CCTTCTTGGGGTCAGCACAG-3′	236	64	
	Ccl4	5′-ACCCTCCCACTTCCTGCTGT-3′	5′-GGGCAGGAAATCTGAACGTG-3′	301	64	
	Ccl5	5′-ATATGGCTCGGACACCACTC-3′	5′-GGGAAGCGTATACAGGGTCA-3′	242	66	
	Ccr1	5′-GGGAGTTCACTCACCGTACC-3′	5′-GATTGTAGGGGGTCCAGAGG-3′	243	62	35
	Ccr2	5′-GCCATGCAGGTGACAGAGAC-3′	5′-AGACCTCTTGCTCCCCAGTG-3′	214	66	35
	Ccr3	5′-GAAGTCCAGGTGTGGTGCTG-3′	5′-GGTATGTGAGGGCCGGTAGA-3′	183	66	35
	Ccr5	5′-CTACCACACCGGGACTGTGA-3′	5′-GCCTGGAACACAGAGAGCAG-3′	275	64	35
	Il6	5′-CCGGAGAGGAGACTTCACAG-3′	5′-GGAAATTGGGGTAGGAAGGA-3′	421	62	
	L19	5′-CTGAAGGTCAAAGGGAATGTG-3′	5′-GGACACAGTCTTGATGATCTC-3′	196	60	
	Snap25	5′-GAGATGCAGAGGAGGGCTGAC-3′	5′-GCTGGCCACTACTCCATCCTG-3′	309	62	
	Tlr2	5′-TTGCTCCTGCGAACTCCTAT-3′	5′-CAATGGGAATCCTGCTCACT-3′	354	60	
	Tlr4	5′-ACCTGGCTGGTTTACACGTC-3′	5′-CAGGCTGTTTGTTCCCAAAT-3′	455	58	
Human	β-actin	5′-CTACAATGAGCTGCGTGTGG-3′	5′-TAGCTCTTCTCCAGGGAGGA-3′	450	58	33
	Tlr2	5′-GATGCCTACTGGGTGGAGAA-3′	5′-GAATGAGAATGGCAGCATCA-3′	256	62	37
	Tlr4	5′-CCATAAAAGCCGAAAGGTGA-3′	5′-CAGGGCTTTTCTGAGTCGTC –3′	265	62	37

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, GGCAUUAAGUCUCCGGAAUtt (sense) and AUUCCGGAGACUUAAUGCCtt (antisense);

TLR4, GCAUCUAUGAUGCAUUUGUtt (sense) and ACAAAUGCAUCAUAGAUGCtt (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 CO_2 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 that diameter, 10000 IU HCG (Gonatropin; Teikoku-zouki) or 600 μg GnRH-agonist was administered; 35 hours later, oocytes were retrieved under ultrasonographic guidance. The oocytes underwent conventional IVF for 5 hours, before being transferred to 500 μl P-1 medium (preimplantation-1 medium; Irvine Scientific) under mineral oil with 10% (v/v) human serum at 37°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. After 12 hours of culture, the formation of pronuclei was observed. The medium after conventional IVF was kept below –80°C before Bio-Plex protein array analysis using the Human Cytokine 9-Plex Panel (BioRad), as described below. All patients gave written informed consent to participate in this study.

RT-PCR analyses

Total RNA was obtained from cumulus cells or granulosa cells using the RNAeasy Mini Kit (Qiagen Sciences, Germantown, MD), according to the manufacturer's instructions. Real-time or quantitative (Q) RT-PCR analyses were performed as described previously (Shimada et al., 2007). Briefly, total RNA was reverse transcribed using 500 ng poly-dT (Amersham Pharmacia Biotech, Newark, NJ) and 0.25 U avian myeloblastosis virus-reverse transcriptase (Promega, Madison, WI) at 42°C for 75 minutes and 95°C for 5 minutes.

For real-time PCR analysis, cDNA and primers were added to the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) to give a total reaction volume of 15 μl. PCR reactions were then performed using the iCycler thermocycler (Bio-Rad). Conditions were set to the following parameters: 10 minutes at 95°C, followed by 45 cycles each of 15 seconds at 95°C and 1 minute at 60, 62 or 64°C. Specific primers were selected and analyzed as indicated in Table 1.

For Q RT-PCR analysis, specific primers pairs, dNTP (Promega), *Taq* polymerase and Thermocycle buffer (Promega) were added to PCR mixture. cDNA products were resolved on 2% (w/v) agarose gels.

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 Hose, 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 culture, media samples were collected and cytokines present in the media were analyzed with the Bio-Plex Protein Array System (BioRad) using the Bio-Plex Mouse Cytokine 23-Plex Panel, including antibodies for interleukin (IL) family members [IL1α, IL1β, IL2, IL3, IL4, IL5, IL6, IL9, IL10, IL12 (p40), IL12 (p70), IL13, 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 IL1β, IL6, IL12 (p70), IL17, Eotaxin (CCL11), GCSF, MCP1 (CCL2), MIP1β (CCL4), and RANTES (CCL5), according to our previous report (Shimada et al., 2007).

Table 2. List of antibodies used for western blotting

Antibody	Company	Dilution
β-Actin (AC74)	Sigma	1:10,000
CCR3	Anaspec, San Hose, CA	1:250
ERK 1/2 (p44/p42 MAP kinase, 9102)	Cell Signaling, Boston, MA	1:1000
pERK1/2 (phospho-p44/p42 MAPK, E10, 9106)	Cell Signaling	1:2000
IκB-a (L35A5, 4814)	Cell Signaling	1:2000
IRF3	Cell Signaling	1:1000
MYD88	eBioscience, San Diago, CA	1:1000
NF-κB (3034)	Cell Signaling	1:1000
phospho-NF-κB p65 (Ser536, 3031)	Cell Signaling	1:1000
phospho-p38MAPK (28B10, 9126)	Cell Signaling	1:2000
Toll-like receptor 2 (2229)	Cell Signaling	1:1000
Toll-like receptor 4 (IMG578A)	IMGENEX, San Diago, CA	1:500
phospho-Tyr (P-Tyr-100, 9411)	Cell Signaling	1:5000

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 Iκβ 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

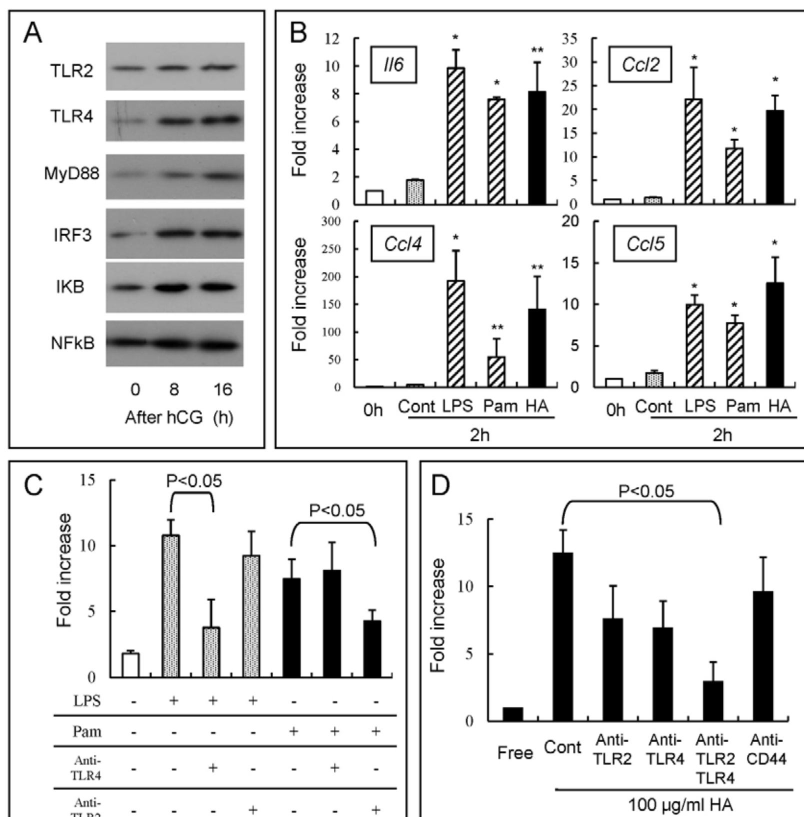

Fig. 1. TLR2 and TLR4 expressed on cumulus cells of ovulated COCs are functional, and both receptors can recognize HA fragments. (**A**) Kinetic changes in the expression of TLRs and related molecules in cumulus cells of COCs during the ovulation process. Results in each panel are representative of two 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.

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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

	Control	LPS	Pam3Cys	Hyaluronan fragment
IL1α	6.34±1.51	47.93±5.33	60.9±5.07	7.02±5.69
IL1β	–	35.01±8.15	78.04±6.66	15.3±2.54
IL6	9.16±2.33	344.84±28.62	472.18±52.63	284.35±18.72
IL9	–	72.23±30.51	65.11±6.25	–
IL12 (p40)	0.08±0.02	36.14±6.94	28.69±3.99	33.33±5.11
IL12 (p70)	52.4±10.36	139.18±11.56	196.32±21.68	27.87±6.33
GCSF	17.61±5.65	173.96±35.63	265.59±32.83	26.71±3.75
MCP1	23.51±3.89	468.82±55.88	506.21±42.68	255.82±20.63
MIP1α	5.13±1.17	182.78±30.42	404.74±25.88	2.85±0.06
MIP1β	11.07±2.20	1082.73±105.36	927.3±62.16	678.56±72.39
RANTES	24.19±5.26	1368.25±256.82	474.58±48.67	674.24±51.78

Ovulated COCs (50 COCs per/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 Iκβ-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 Iκβ 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 Iκβ 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.

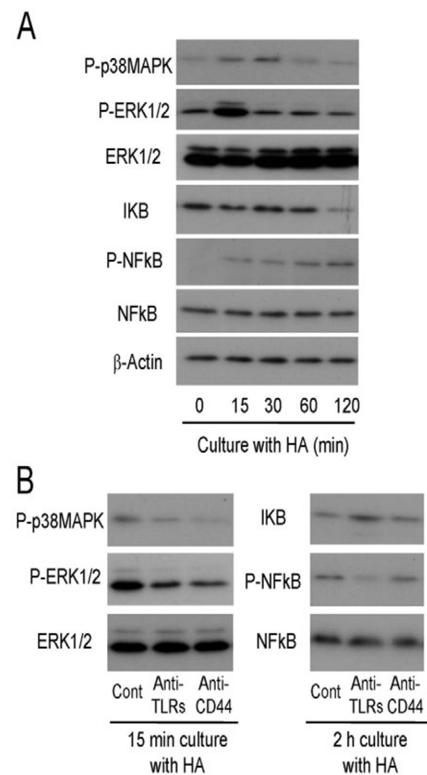

Fig. 2. HA fragments induced the TLR2-, TLR4- and CD44-targeted signal transduction pathways. (**A**) Time-dependent changes of the activation of p38MAP kinase, ERK1/2 and the NFκB pathway in cumulus cells of ovulated COCs cultured with 100 μg/ml of HA fragments. Results in each panel are representative 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 (Iκβ and NFκB). Results in each panel are representative of two separate experiments.

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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 Iκβ and the phosphorylation of NFκB in cumulus cells of COCs, responses that were reversed by anti-TLR2 and anti-TLR4 neutralizing antibodies (Fig. 3B).

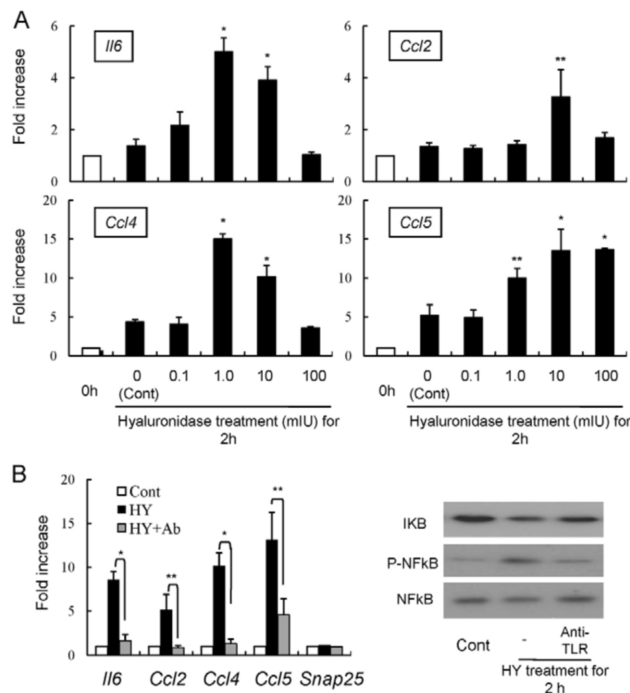

Fig. 3. The effects of hyaluronidase (HY) treatment on gene expression and NFκB pathway activation in cumulus cells of ovulated COCs. (**A**) Dose-dependent effects of HY on the expression of cytokine and chemokine mRNA. Ovulated COCs were treated with 0 to 100 mIU/ml of HY for 2 hours. The 0 hour value 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.

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 of 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 CCR1 and partly to CCR5, and CCL4 selectively activates CCR5. All of receptors (CCR1, CCR2, CCR3 and CCR5) were activated by CCL5 (Charo and Ransohoff, 2006). The CCR receptors are members of the G-protein coupling receptor family that induce phospholipase C to increase Ca^{2+} and PKC activation in cytoplasm (Meyer et al., 1996). In sperm, the Ca^{2+}-PKC pathway is involved in capacitation (Rotem et al., 1992), suggesting that the COC-secreted CC chemokine family might play an important role in sperm capacition during the fertilization process. The RT-PCR analysis shown in Fig. 5A shows the expression of *Ccr1*, *Ccr2*, *Ccr3* and *Ccr5* mRNA in sperm collected from the cauda epididymis. The positive immunofluorescent signals observed following the use of an anti-CCR3 antibody localized CCR3 to luminal region of the testicular seminiferous tubules, and, at higher magnification, showed that CCR3 was detected near the tail of spermatozoa; however these signals were very weak and a few cells

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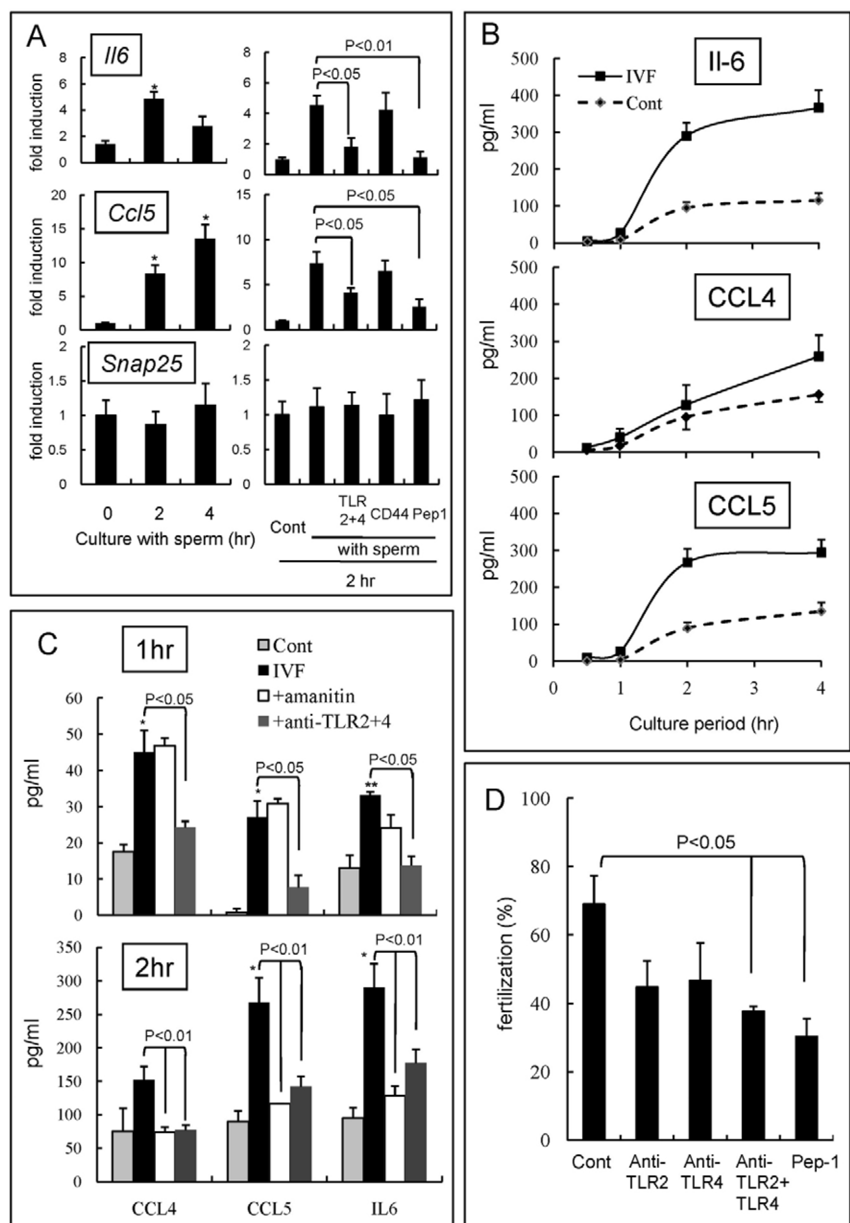

Fig. 4. The roles of TLR2/TLR4 in regulating the expression and secretion of cytokines/chemokines during the in vitro fertilization process. (**A**) The expression of *Il6*, *Ccl5* and *Snap25* mRNA in cumulus cells of COCs cultured with sperm. Ovulated COCs were cultured with sperm for 2 or 4 hours. 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. **P*<0.01 (significant difference observed compared with that at 0 hour). Some COCs were cultured with anti-TLR2 and anti-TLR4 neutralizing antibodies (TLR2+4), anti-CD44 antibody (CD44), or HA-blocking peptide (Pep-1) for 30 minutes, and then further cultured with the sperm for 2 hours. The Cont value was set as 1, and the data are presented as fold increase. Values are mean±s.e.m. of three replicates. Cont, ovulated COCs cultured for 2 hours without sperm. (**B**) The secretion of IL6, CCL4 and CCL5 from COCs during the fertilization process. IVF, COCs cultured with sperm for up to 4 hours; Cont, ovulated COCs cultured for up to 4 hours without sperm. (**C**) Effects of the mRNA synthesis inhibitor, or anti-TLRs neutralizing antibodies, on the secretion of cytokines and chemokines from COCs. COCs were pre-cultured for 30 minutes with neutralizing antibodies (+anti-TLR2+4) or 10 μg/ml of α-amanitin (+amanitin), and then further cultured with sperm for 1 or 2 hours. Culture with sperm (IVF) significantly increased secreted levels as compared with those in the control (**P*<0.01; ***P*<0.05). Cont, ovulated COCs cultured for 1 or 2 hours without sperm. (**D**) Effects of anti-TLR2/TLR4 neutralizing antibodies or Pep-1 on fertilization. COCs were pre-cultured for 30 minutes with Pep-1 or neutralizing antibodies (anti-TLR2 antibody, anti-TLR4 antibody, or both), and further cultured for 12 hours with sperm. Twelve hours after insemination, oocytes were checked the formation of pronuclei under a phase-contrast microscope. Data are presented as the percentage of oocytes fertilized. Cont, ovulated COCs cultured with sperm for 12 hours.

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

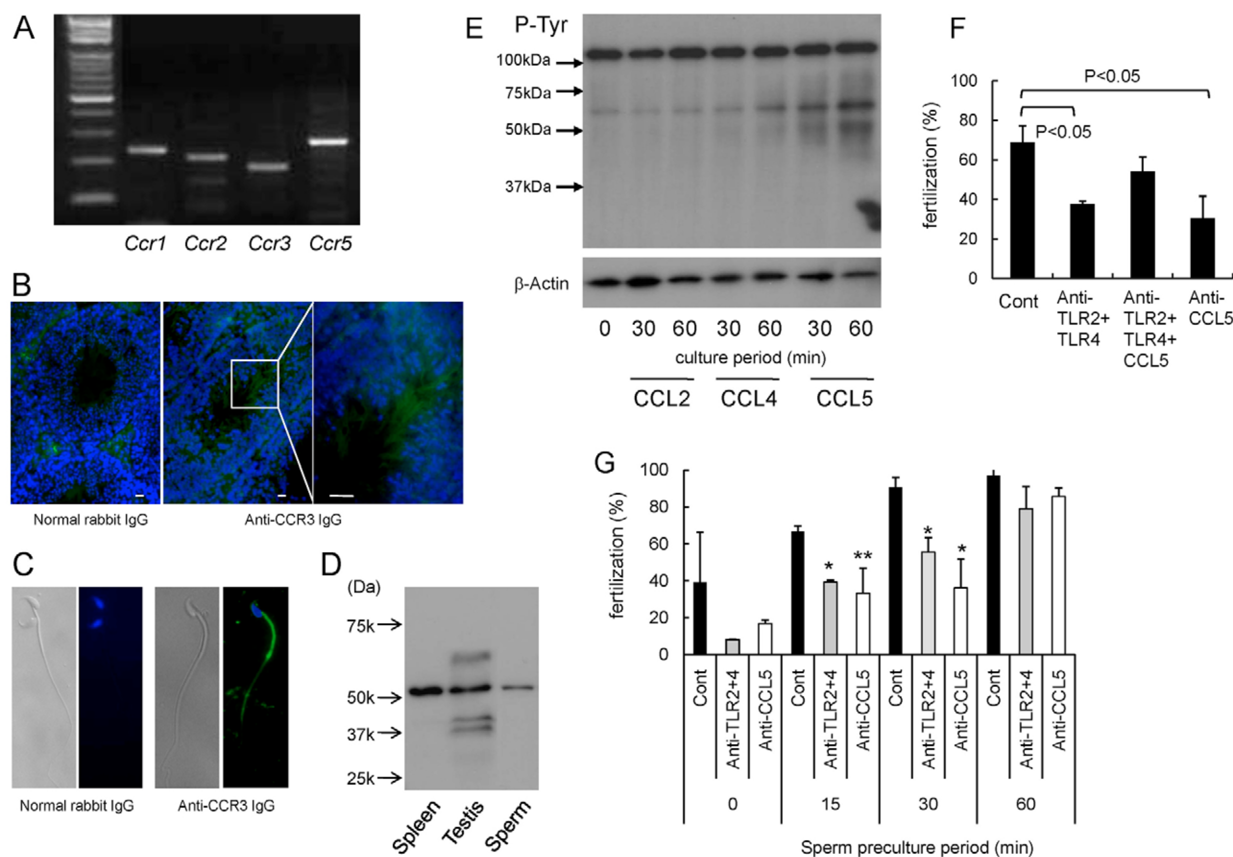

Fig. 5. Sperm express chemokine receptors required for sperm capacitation during the fertilization process. (**A**) The expression of *Ccr1*, *Ccr2*, *Ccr3* and *Ccr5* mRNA in sperm was determined by RT-PCR. (**B**,**C**) The localization of CCR3 in testicular seminiferous tubes (B) and sperm (C). Blue, DAPI staining of nuclei; green, FITC signal localizing the anti-CCR3 antibody. Scale bar: 10 μm. As a negative control, the slides were incubated with normal rabbit IgG and then reacted with secondary antibody. (**D**) Western analysis of CCR3 using the same primary antibody that was used for immunofluorescence. (**E**) The induction of protein tyrosine phosphorylation in sperm by CCL2, CCL4 or CCL5. Sperm collected from cauda epididymi were cultured with 100 pg/ml of CCL2, CCL4 or CCL5 for 30 or 60 minutes. Tyrosine phosphorylation (P-Tyr) was detected by an anti-phospho-Tyr antibody. (**F**) CCL5 regulates sperm penetration to oocytes. COCs were pre-cultured for 30 minutes with anti-TLR2 + anti-TLR4 antibodies (Anti-TLR2+TLR4), or with anti-CCL5 antibody (Anti-CCL5), and further cultured for 12 hours with sperm. In some cases, CCL5 (100 pg/ml) was added to the fertilization medium (+CCL5). Data are presented as the percentage of oocytes fertilized. Cont, ovulated COCs cultured with sperm for 12 hours. (**G**) The effects of the pre-culture period of sperm on oocyte fertilization in vitro. COCs were pre-cultured for 30 minutes with anti-TLR2 + anti-TLR4 antibodies (Anti-TLR2+TLR4), or with anti-CCL5 antibody (Anti-CCL5), and further cultured for 12 hours with sperm. The sperm were collected from the cauda epididymis, and then cultured for 0, 15, 30 or 60 minutes. Data are presented as the percentage of oocytes fertilized.

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).

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 *Tlr2* and *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).

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

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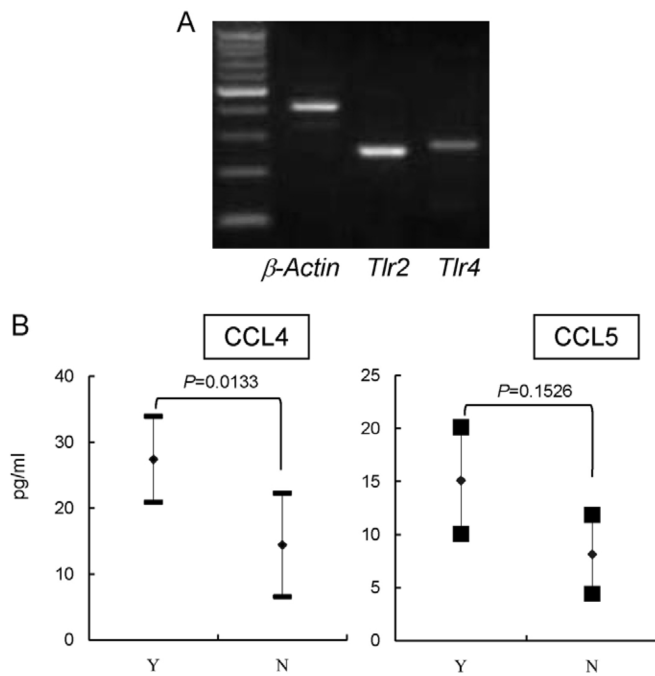

Fig. 6. The expression of *Tlr2* and *Tlr4* mRNA and the role of cytokines/chemokines in human fertility. (**A**) The expression of *Tlr2* and *Tlr4* mRNA in cumulus cells of human COCs. The cumulus cells were recovered from COCs after in vitro fertilization. (**B**) The relationship between the amount of secreted CCL cytokines and fertilization 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.

(LPS), the expression of *Il6* and *Tnfα* was induced. However, the endogenous ligand(s) for TLR2/TLR4 and the physiological role of this signaling cascade in ovulated COCs remained unclear. Because cumulus cells produce and are surrounded by a HA-rich extracellular matrix and because HA fragments have been shown recently to activate TLR2 and TLR4 (Termeer et al., 2002; Jiang et al., 2005), we hypothesized that HA fragments generated during matrix degradation might act as an endogenous ligand for TLR2 and/or TLR4 that are present in cumulus cells of ovulated COCs. Herein, we provide the first evidence that TLRs can play a functional role in the, at least in vitro, fertilization process. Notably, we show that hyaluronidase treatment and culture with sperm (used as a biological source of hyaluronidase) activate TLR2/TLR4 on cumulus cells. Activated cumulus cells, but not sperm itself, then release specific cytokines capable of enhancing sperm capacitation and fertilization. Thus, a functional regulatory loop appears to be operative between sperm and the ovulated COCs.

Specifically, the results presented here document that exposure of ovulated COCs to HA fragments induced the phosphorylation of p38MAP kinase, ERK1/2 and NFκB, and the expression of specific genes (*Il6*, *Ccl2*, *Ccl4* and *Ccl5*) in cumulus cells. This response was similar to that observed when ovulated COCs were exposed to LPS, a known ligand of TLR4 (Shimada et al., 2006), indicating that HA fragments could serve as ligands for cumulus cell TLRs. The ability of HA fragments to active these signaling cascades and to induce the expression of specific cytokines in cumulus cells was selectively mediated by TLR2 and TLR4, as TLR2 and TLR4 neutralizing 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 2×10^3 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; Muciaccia 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 phospho-tyrosine were detected in extracts of the CCL5 treatment group.

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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 secret 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.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/11/2001/DC1

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DEVELOPMENT

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