Targeting interleukin-6 receptor inhibits preterm delivery induced by inflammation

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ABSTRACT: Intrauterine infection is still a common trigger of preterm delivery (PTD) and also a determinant risk factor for the subsequent development of neurodevelopmental abnormalities in neonates. In this study, we examined the expressional pattern of various inflammatory cytokines such as interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α) in placentae complicated with severe chorioamnionitis (CAM) and found that IL-6 is mainly expressed in macrophages in villous mesenchyme by immunohistochemical analysis with anti-CD-68 antibody. Using an experimental lipopolysaccharide (LPS)-induced PTD model, the therapeutic potential of targeting this cytokine was investigated. Anti-IL-6 receptor antibody (MR16-1) was delivered 6 h before LPS treatment. Mice in the MR16-1 group had a significantly lower rate of PTD (17%) than in the controls (53%, P = 0.026). As a result, MR16-1 treatment significantly prolonged the gestational period (control; 18.4 ± 1.7d, MR16-1; 19.8 ± 1.5d, P = 0.007) without any apparent adverse events on the mice and their pups. In primary human amniotic epithelial cells, pretreatment with a humanized anti-human IL-6 receptor antibody, tocilizumab, significantly inhibited the production of prostaglandin E2 induced by IL-6. In conclusion, IL-6 was strongly expressed mainly in macrophages in villous mesenchyme in placentae complicated with CAM. Anti-IL-6R antibody significantly decreased the rate of PTD in LPS-induced inflammatory model in mice, and inhibited PGE2 production from human primary amniotic epithelial cells. Targeting IL-6 signaling could be a promising option for the prevention of PTD and needs to be further explored for future clinical application.

Key words: interleukin-6 / macrophage / tocilizumab / preterm delivery / chorioamnionitis

Introduction

Preterm delivery (PTD) (before 37 completed weeks in humans) remains a significant public health problem all over the world. In developed countries, PTD occurs in up to 10% of all pregnancies and is associated with 75% of infant mortality and 50% of long-term neurologic handicaps (Berkowitz and Papiernik, 1993). Currently, beta-adrenergic agonists (e.g. ritodrine, terbutaline) and magnesium sulfate are considered to be the first-line tocolytic agents of choice when indicated; however, tocolysis with these agents is rarely successful beyond 24–48 h because current medications do not alter the fundamentals of labor activation (Simhan and Caritis, 2007).

Although several pathological causes have been suggested, intrauterine infection and/or inflammation, represented by chorioamnionitis (CAM), is the most firmly established trigger of early PTD. Ascending bacteria from the vagina reach the pregnant uterus via the cervix to provoke an inflammatory reaction in the decidua, chorion, amnion, amniotic fluid and ultimately the fetus (Arcuri et al., 2009). At the inflammatory site, leukocytes such as neutrophils or macrophages are recruited and activated and eventually secrete proinflammatory cytokines including tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, IL-6 or IL-8 in response to bacteria or bacterial products (e.g. lipopolysaccharide (LPS)) (Gomez-Lopez et al., 2010). These cytokines initiate the synthesis of secondary mediators, e.g. prostaglandins (PGs) and matrix metalloproteinases (MMPs), which contribute to preterm labor, preterm premature rupture of membranes (pPROM) and finally to the onset of PTD (Arcuri et al., 2009). In addition, recent evidence strongly suggests that the poor neurological outcomes of children delivered preterm are not solely dependent on their gestational age and weight at birth (Draper et al., 1999). The increased expression of proinflammatory cytokines...
cytokines has been identified in the human infant brain complicated with periventricular leukomalacia (PVL), indicating that these mediate infection-induced fetal or neonatal brain injury (Pang et al., 2006). Therefore, targeting these cytokines could be a new promising approach to reduce not only PTD but its related neonatal disorders, although all tocolytic agents currently available are utilized just to block PG synthesis or reduce uterine contractility.

Various clinical data have shown that CAM leads to a rise of several cytokines in amniotic fluid, fetal cord blood and maternal serum (Murtha et al., 2007). Among these, IL-6 is a well-known infection inflammation marker and has been reported to be significantly elevated in maternal serum, amniotic fluid and vaginal secretions in CAM-complicated patients (Sorokin et al., 2010; Gulati et al., 2012). Recently, Taylor et al. assessed what kind of inflammation biomarkers detected in the vaginal fluid are the most informative for identifying PTD and showed that the elevated IL-6 concentration at midtrimester was associated with increased odds of spontaneous PTD at <35 weeks as well as PTD accompanied by CAM, and had the greatest sensitivity for detecting these two PTD subtypes (Taylor et al., 2013). However, these reports concerning IL-6 predominantly focused on its potential as a predictor of CAM or PTD even in the absence of positive amniotic fluid culture (Cobo et al., 2009), whereas little has reported about the therapeutic potential of targeting this cytokine, although IL-6 appears to play the pivotal role in the etiology of these diseases. Recently, anti-cytokine therapies have emerged in the clinical setting, as is evidenced by the success of tocilizumab (Chugai Pharmaceutical, Tokyo, Japan), an anti-human IL-6 receptor antibody (humanized from mouse anti-human IL-6 receptor), to alleviate Castleman’s disease and rheumatoid arthritis (Nishimoto et al., 2007).

With these in mind, we first examined the expressional patterns of various inflammatory cytokines in clinical CAM-complicated placentae and found that macrophages in placentae will produce IL-6. The potential of anti-IL-6 receptor antibody as a therapeutic agent for PTD was evaluated using an inflammatory PTD mouse model. Antagonizing IL-6 receptor significantly prolonged the gestational period without any apparent adverse events on the mice and their pups. In primary human amniotic epithelial cells, pretreatment with tocilizumab inhibited the production of PGE2 induced by IL-6. Thus, this present study provides new insights into possible future treatment strategies to overcome the poor outcome of early PTD and its complicated neonatal disorders.

**Materials and Methods**

**Materials**

Antibodies against TGF-β, interleukin-1β (IL-1β) (C20), IL-6 (R19 and R49L), IL-8 (NYR-HIL8), CD68 (PG-M1) and Cytokeratin 18 (RGE53) were all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against valentine (R28), cyclooxygenase2 (COX2) and β-actin were from Cell Signaling Technology (Boston, MA, USA). Anti-TNF-α (4C6H6) antibody was from Cosmobio Co. Ltd. (Tokyo, Japan). Lipopolysaccharides (LPS) from Escherichia coli (serotype 055: B5) and IL-8 (2 μg/ml) at 1:50 for 1 h at room temperature, followed by the application of DAB solution, and further incubated with anti-IL-6 antibody at 1:50 (4 μg/ml) for 16 h at 4°C, followed by the application of HistoGreen (Ab Cys S.A., Paris, France) staining solution in accordance with the manufacturer’s protocol.

**Placental tissue collection**

Placental tissue sections were obtained from patients with CAM-complicated PTDs (n = 10) and idiopathic gestational age-matched preterm control subjects (n = 6) at Osaka Medical Center and Research Institute for Maternal and Child Health (Osaka, Japan) between 2005 and 2010. Written informed consent was obtained from each patient and the collections of placentae were approved by the ethics committees of the institute. All the placental specimens were histologically examined by qualified pathologists (M.N. and A.K.). Patients complicated with CAM were clinically diagnosed with maternal fever (38°C), uterine tenderness, foul-smelling amniotic fluid at the time of the speculum examination, maternal tachycardia (100 beats/min) and fetal tachycardia (160 beats/min) (Romero et al., 1993). CAM-complicated placentae were also histologically diagnosed based on the criteria of Naeye (Naeye, 1983), as having >10 neutrophils per high-power field in the subchorionic space, chorio or placental plate. All cases showed a histological presentation of sub-acute necrotizing funisitis.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded tissue sections were sliced at a thickness of 4 μm. The sections were deparaffinized in xylene and dehydrated with 100% ethanol before antigen unmasking was performed by boiling the sections in Target Retrieval Solution (pH 6.0) (Dako, Glostrup, Denmark). After being placed in 3% H2O2 and blocked with Blocking Solution (Dako), the sections were incubated with the primary antibodies against TNF-α, TGF-β (4 μg/ml), IL-1β (4 μg/ml), IL-8 (4 μg/ml) and IL-8 (2 μg/ml) at 1:50 for 1 h at room temperature. After washing with 0.15 mol/l Tris-buffered saline (TBS) (pH 7.6), the slides were stained using Histofine Simple Stain MAX-PO (MULTI) (Nichirei Bioscience, Tokyo, Japan), followed by the application of DAB solution until the desired stain intensity developed, and then counterstained with Mayer’s hematoxylin. For double staining, sections were first incubated with anti-CD68 antibody at 1:50 for 1 h at room temperature, followed by the application of DAB solution, and further incubated with anti-IL-6 antibody at 1:50 (4 μg/ml) for 16 h at 4°C, followed by the application of HistoGreen (Ab Cys S.A., Paris, France) staining solution in accordance with the manufacturer’s protocol.

**Animal experiments**

C3H/HeN female mice and B6D2F1 male mice were purchased from Charles River Japan (Yokohama, Japan). The animals were housed individually under controlled conditions at 25°C and 60% relative humidity on a 12-h light/12-h dark cycle. C3H/HeN female null-para mice (7–12 weeks old) were pair-mated with B6D2F1 male mice (7–30 weeks old). The day when a vaginal plug was detected was designated as 0.5 day of gestation. All pregnant mice delivered their pups on Day 19 or 20. PTD was defined as delivery before 18.5 days of gestation because pups born before Day 18.5 could not survive >24 h after birth. For mouse models of endotoxin-induced PTD, on Day 15.5 of gestation, 100 μg/kg LPS dissolved in 200 μl of 0.9% saline was injected twice intraperitoneally, with a 3-h interval between injections (14:00 and 17:00 h). In our initial dose–response series, 100 μg was the lowest dose of LPS that reproducibly induced PTD, without significant morbidity or mortality, in gravid C3H/HeN females, based on the established method reported by Kaga (Kaga et al., 1996). For the prevention study, 12 mg/kg MR16-1 diluted in 200 μl PBS was administered intraperitoneally 24 h before LPS treatment (at 17:00 on Day 14.5 of gestation). An equivalent volume of non-immune mouse IgG and PBS alone were administrated as ‘Control IgG’ and ‘Mock’, respectively. RU486 dissolved to a concentration of 10 mM in absolute ethanol was diluted in 0.9% saline solution. Then, 150 μg/350 μl of RU486 was injected...
subcutaneously on Day 16.0 of gestation to induce non-infectious premature delivery. All protocols were in accordance with the National Institutes of Health Guidelines and were approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Medicine.

Measurement of IL-6 concentration
Murine maternal blood samples were obtained from a tail vein at 13:00 (before the initial LPS injection) and 18:00 (1 h after the second LPS injection) on Day 15.5; 100 μl samples were collected each time. The blood samples were allowed to clot for 2 h at room temperature or overnight at 4 °C, and centrifuged for 20 min at 335g. Plasma was removed and stored at −80 °C until assayed. The concentration of IL-6 was measured using the Quantikin Mouse IL-6 Immunoassay (R&D Systems, Inc.) as recommended by the manufacturer.

Quantitative RT–PCR of murine amnion
Murine amnions were obtained by Cesarean section 12 h after LPS treatment (at 5:00 on Day 16.5). RNA was extracted using TRizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. cDNA was synthesized using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Real-time quantitative RT–PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) with the following probes from Applied Biosystems: murine prostaglandin-endoperoxide synthase 2 (ptgs2: Mm00478374_m1) and murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Mm03302249_g1). Relative levels of mRNA gene expression were calculated using the 2−ΔΔCT method as described previously (Livak and Schmittgen, 2001).

Isolation of human primary amniotic epithelial cells
Human primary amniotic epithelial cells (hAECs) were obtained based on the method reviewed by Parolini et al. (Parolini et al., 2008). Briefly, amniotic membranes were obtained from the placenta of patients who underwent uncomplicated Cesarean sections at term at Osaka University Medical Hospital (Osaka, Japan) between 2009 and 2012. None of the patients from whom specimens were obtained was in labor. Informed consent was obtained from each patient for the usage of the samples before the operation and the collections of amnions were approved by the ethics committees of the institute. Amniotic membranes were washed thoroughly with ice-cold PBS, cut into small pieces (1–2 mm) and incubated in 0.05% trypsin at 37 °C for 1 h. Thereafter, the pieces were placed in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution (Invitrogen) shaken for 20 s and strained through 40 μm nylon mesh. The elute was collected and centrifuged at 241g for 10 min and the precipitate was cultured as hAECs in DMEM with 10% FBS. Cells were grown in a standard 95% air-5% CO2 incubator at 37 °C. Of these were utilized in this study.

RT–PCR analysis
Total RNA was extracted from hAEC cells using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized from 1 μg RNA using a ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) with random primers. The cDNA templates were PCR-amplified with Taq PCR master mix (Qiagen, Valencia, CA, USA) containing 1 mM each of dATP, dCTP, dGTP and dTTP, and 2.5 U Taq DNA polymerase, and each specific primer at 0.2 μM under the following conditions: 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The products were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining and ultraviolet illumination. The primers were designed based on the previous report by Nakanishi et al. (2004). The primer sequences and the expected PCR products are as follows: IL-6-sense: 5′-TACGGCCGCCAACACAGAC-3′, IL-6-antisense: 5′-GGTGGCATTTGTTGTCG-3′, size 408 bp; IL-6R-sense: 5′-CATGCTTGTTCGTAAGGTTC-3′, size 150–161; because this primer annealed to the region splicing out from IL-6R, thus sIL-6R transcript could not be amplified), IL-6R-antisense: 5′-GGCGAATCCGAGGACACT-3′ (bases 1419–1438; size 80 bp; IL-6R-sense: 5′-TTGTGGTTGG-3′, IL-6R-antisense: 5′-GGGTCCACCCAGCGACTATC-3′ (bases 1840–1859, same as IL-6-antisense), size 280 bp; sIL-6-sense: 5′-GCCAACCCACCAAGGACACT-3′ (bases 615 bp; β-actin-sense: 5′-GGTTGACATTTGGAGCTG-3′, β-actin-antisense: 5′-GCCTACGGAAGGAGCTG-3′, size 376 bp. The sequences of cDNA were referred to GenBank (accession no. NM_000600 for IL-6, NM_012830 for IL-6R and NM_001101 for β-actin).

Quantification of PGE2
4 × 105 of primary hAECs were plated on 12-well plates and allowed to attach overnight. The cells were then starved in serum-free media with 10 μg/ml tcoliculzumab or the equivalent volume of control IgG for 24 h. Thereafter, the cells were stimulated with 100 ng/ml human IL-6 plus 60 ng/ml human soluble IL-6 receptor for 24 h. Conditioned culture media were collected and stored at −80 °C until analysis. Prostaglandin E2 (EIA Kit-Monoclonal (Cayman Chemical Company, Ann Arbor, MI) was used to determine the concentration of PGE2 in accordance with the manufacturer’s protocol.

Western blotting
1 × 106 of primary hAECs were plated on 6-well plates and stimulated as described above. Thereafter, cells were lysed with 1 × Cell Lysis buffer (Cell Signaling, Beverly, MA, USA) (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na3EDTA, 1 mMEGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 mM PMSF). Lysates were separated by 5–20% gradient SDS–PAGE and transferred to PVDF membranes followed by incubating with the primary antibodies (cytotoxicogenase2 (COX2); 1:500 in 5% BSA, actin; 1:10.000 in 5% milk) and then with the corresponding secondary horseradish peroxidase-conjugated IgG. The proteins were visualized using the ECL system (PerkinElmer Life Science, Boston, MA, USA).

Statistical analysis
Statistical analysis was performed using Excel 2007 (Microsoft) with add-in software Statcel2 (OMS, Tokyo, Japan). The significance of differences between means was evaluated by one factor ANOVA, followed by the Tukey–Kramer method or Student’s t-test. Bar graph data in Figures are expressed as the means ± SD. Statistical significance was set at P < 0.05.

Results
IL-6 is expressed in villous mesenchymal cells
Since inflammatory and related cytokines such as IL-1, IL-6, IL-8 and TNF-α have been proved to be substantially increased in the amniotic fluid of women complicated with CAM (Saji et al., 2000), we first examined the expression pattern of those cytokines and the types of cells that produce them in CAM placenta. Using 10 cases of severe CAM placenta, immunohistochemical analyses were performed. Representative images are shown of the placenta of a patient who terminated her...
pregnancy at 24 weeks of gestation complicated by CAM (Fig. 1). TNF-α was expressed in trophoblasts (Fig. 1A) and TGF-β was seen in trophoblasts as well as mesenchymal villi (Fig. 1B). IL-1β was also seen in mesenchymal villi (Fig. 1C). In contrast, IL-6 staining was seen mainly in villous mesenchymal cells, suggesting that these cells were recruited in response to bacterial infection (Fig. 1D). Strong IL-6 staining was also seen in villous mesenchymal cells of the placenta of other patients who terminated their pregnancy complicated by CAM, whereas decidual cells were not stained (Supplementary data, Fig. S1A and B). A neutrophil chemokine, IL-8 (Fig. 1E), was not expressed in placental villi from this study.

IL-6 is secreted from macrophages in chorionic villi and plates in CAM-complicated placenta

Since IL-6 is expressed in mesenchymal cells in villi and these cells displayed a macrophage-like morphology, we double-stained the sections with IL-6 (Histogreen: green) and CD68, a representative marker of macrophages (DAB: brown) (Fig. 2A–D). Placental tissue sections from patients with CAM-complicated PTDs (n = 10) and idiopathic gestational age-matched preterm control subjects (n = 6) were analyzed and the clinical features of the patients are summarized in Table I. Among 10 patients with CAM-complicated PTDs, 6 delivered vaginally and 4 underwent Cesarean sections. Mean gestational ages of CAM complicated and control pregnancies were 26.4 weeks and 26.5 weeks, respectively. Five representative images of placental villi and chorionic plates were taken randomly and the number of CD68-positive cells per field (×200 magnification) was counted. The average number of CD68-positive cells in placentae complicated by CAM was 99 ± 26/field and was significantly higher than that of the control 60 ± 18 (P < 0.05). Furthermore, 88% of CD68-positive cells showed IL-6-positive staining in CAM placentae, while 48% of CD68-positive cells expressed IL-6 in control placentae (P < 0.01) (Fig. 2H). The average number of CD68-positive cells in placentae of patients who delivered vaginally was 99 ± 30/field (89% of CD68-positive cells expressed IL-6) and that of patients who underwent Cesarean sections was 100 ± 22/field (88% of cells expressed IL-6). In addition, the average number of CD68-positive cells in placentae of patients who delivered < 28 weeks of gestation was 96 ± 31/field (89% of cells expressed IL-6) and that of patients who delivered later than 28 weeks of gestation was 119 ± 22/field (85% of cells expressed IL-6). Therefore, no significant differences were seen between the modes of delivery or at these different gestations in this study. These immunohistochemical findings indicated that, in response to bacterial infection, macrophages that produce IL-6 are recruited and overexpressed in placental villi and chorionic plates, strongly suggesting the involvement of IL-6 in the pathogenesis of infection-related PTD.

MR16-1, a rat anti-IL-6 receptor antibody, prevented PTD in a LPS-induced PTD mouse model

Since IL-6 is strongly expressed in CAM-complicated placentae, indicating the essential role of IL-6 in infection-related PTD, we were encouraged to examine whether anti-cytokine therapy against IL-6 signaling could prevent PTD using a LPS-induced PTD mouse model. C3H/HeN mice, which were pair-mated with B6D2F1 male mice, are...
known to deliver preterm in response to low-dose LPS, as we and others previously reported (Tahara et al., 2005). First, we tested three different low doses (50, 100, 150 μg/kg) of LPS administration to find an appropriate regimen that induces the highest incidence of PTD without causing any morbidity or mortality of pregnant mice (Supplementary data, Table S1). While at double doses of 50 μg/kg with a 3-h interval, only 22% (8/37) of gravid mice delivered their pups preterm after LPS administration, at a dose of 100 μg/kg, 56% (14/25) of mice delivered preterm without any maternal deaths. At a dose of 150 μg/kg, although the incidence of PTD (71%) was higher than that of 100 μg/kg, 3 of 10 mice died within 24 h. Therefore, we determined to adopt double doses of 100 μg/kg with a 3-h interval to induce PTD in this study and found that LPS administration induced PTD in five of eight mice tested without any maternal deaths. For the prevention study, 12 mg/kg MR16-1, anti-mouse IL-6 receptor antibody, was delivered 24 h before LPS administration. While pretreatment with non-immune control IgG did not significantly affect the rate of PTD induced by LPS (53%; 9 of 17 delivered preterm), pregnant mice pretreated with MR16-1 showed a significantly lower rate of PTD (17%; 3 of 18 delivered preterm) (P = 0.026) (Fig. 3B). Accordingly, MR16-1 treatment significantly prolonged the average gestational period of (control IgG: 18.4 ± 1.7 d, MR16-1: 19.8 ± 1.5 d, P = 0.007) (Fig. 3C). Since MR16-1 was effective in preventing LPS-induced PTD, the effects of LPS on IL-6 were analyzed in murine maternal serum. Serum IL-6 concentration was markedly increased 1 h after LPS administration. MR16-1 pretreatment did not affect the IL-6 concentration compared with control IgG (control IgG: 16.2 ± 13.1 ng/ml, MR16-1: 24.5 ± 17.6 ng/ml, respectively) (Fig. 3D). Amniotic tissues were carefully collected and the relative expression of ptgs2 was analyzed. While LPS administration markedly increased ptgs2 expression in amnions, pretreatment with MR16-1 almost abolished this up-regulation (Fig. 3E), indicating that MR16-1 prevents LPS-induced PTD by the inhibition of ptgs2 up-regulation in amnions.

**Table 1 Clinical characteristics of the patients.**

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aPreterm premature rupture of membranes.

bChorioamnionitis.

cPreterm delivery.

dVaginal delivery.

*cesarean section.

fPregnancy-induced hypertension.

g/Preterm premature rupture of membranes.

hAECs.

iPreterm delivery.

jVaginal delivery.

kCesarean section.

lPregnancy-induced hypertension.

mFetal growth restriction.

nPreterm premature rupture of membranes.

Table 1 Clinical characteristics of the patients.

Tocilizumab, an anti-IL-6 receptor antibody, inhibited PGE2 production of human amniotic epithelial cells in response to IL-6 stimuli

Since antagonizing IL-6 receptor prevents inflammatory-induced PTD in a mouse model, we were further encouraged to analyze whether anti-cytokine therapy targeting IL-6 receptor has the potential to prevent PTD in humans. Tocilizumab, which competitively blocks IL-6 binding to its receptor (IL-6R) and has been successfully used in the clinical setting for Castleman’s disease and rheumatoid arthritis, was kindly provided by Chugai Pharmaceutical (Tokyo, Japan) and used for analysis of the tocolytic effect against primary human primary amniotic cells (hAECs). hAECs were carefully obtained from the placenta of patients who underwent uncomplicated Cesarean sections at term. hAECs...
displayed a cobble-stone like morphology (Fig. 4A) and were cytokeratin-18 positive as well as vimentin negative, as previously reported (Parolini et al., 2008) (Fig. 4B and C). RT–PCR analysis revealed that hAECs did not express either IL-6 or IL-6R, while gp130 was expressed. A human ovarian cancer cell line, SKOV3ip1, was used as a positive control for IL-6 and IL-6R (Fig. 4E). The generation of PGs in amnions is fundamental to both normal and preterm labor. PG synthesis requires the initial conversion of arachidonic acid to PGH2 and cyclooxygenase (COX) catalysis as the first step (Gross et al., 2000). Two COX isoforms (COX-1 and COX-2) have been identified and COX-2 is known to be inducible in response to inflammation, and COX-2 up-regulation was reported to be strongly associated with the onset of PTD, whereas COX-1 has been constitutively detected in many tissue types (Gibb, 1998; Sakai et al., 2001). In hAECs, co-treatment with IL-6 and sIL-6R markedly up-regulated COX-2 expression, while IL-6 or sIL-6R alone did not (data not shown). This up-regulation was almost completely abolished by pretreatment with 10 μg/ml tocilizumab (Fig. 4F). Subsequently, the production of PGE2 was assessed by measuring the concentration in culture media. Co-treatment with IL-6 and sIL-6R for 24 h significantly increased the PGE2 concentration (control IgG: 117.9 ± 25.5 pg/ml, IgG + IL-6 + sIL-6R: 194.1 ± 35.9 pg/ml, P < 0.05), while pretreatment with tocilizumab significantly inhibited this increase (tocilizumab + IL-6 + sIL-6R: 75.2 ± 36.3 pg/ml, P < 0.01) (Fig. 4G). Tocilizumab almost completely suppressed PGE2 production induced by IL-6 stimuli in human amniotic cells, suggesting that anti-cytokine therapy targeting IL-6 signaling has the potential to prevent inflammatory-induced PTD in the clinical setting.

Discussion

PTD rates have continued to increase over the last two decades, even in many developed countries (Slattery and Morrison, 2002) and this remains an important public health concern all over the world. Many different classes of drugs have been used for tocolytic therapy, including beta-adrenergic agonists such as ritodrine and terbutaline, magnesium sulfate, prostaglandin inhibitors, calcium channel blockers and others; however, over the three decades since ritodrine was approved, none of these agents have lived up to the expectation that tocolysis could be maintained for longer than 48 h (Simhan and Caritis, 2007). Based on the latest systematic review and network meta-analysis, prostaglandin inhibitors have the highest probability of being the most effective class for delaying PTD and had the most favorable maternal side effect profile (Haas et al., 2012), although their use needs to be limited in practice because some data indicate a possible association between neonatal...
complications and antenatal use of prostaglandin inhibitors such as premature closure of the ductus arteriosus (Norton et al., 1993). Therefore, we believe there is an urgent need to find a safer and more effective drug to prevent PTD and prolong pregnancy. Although numerous studies have confirmed that inflammatory cytokines such as IL-1, IL-6, or TNF-α play a central role in parturition including PTD, no anti-cytokine therapies have been utilized in the clinical setting. To the best of our knowledge, this is the first study to examine the therapeutic potential of an anti-IL-6 receptor antibody, tocilizumab, as a therapeutic agent for PTD using an inflammatory PTD mouse model and to confirm that antagonizing IL-6 receptor significantly prolonged the gestational period without any apparent adverse events on the mice and their pups. These data provide a requisite step before an anti-IL-6 receptor antibody could be considered for the prevention of PTD in human pregnancy.

Although the etiology of PTD is complex, numerous studies have shown that inflammatory cytokines caused by infection are one of the most important underlying causes. Recently, the Pregnancy Outcomes and Community Health (POUCH) cohort study was designed to investigate pathways to PTD in Michigan (Taylor et al., 2013). In their report, vaginal fluid IL-6 levels at midtrimester had the greatest sensitivity for detecting spontaneous delivery at 35 weeks’ gestation and PTD accompanied by CAM. Although elevated levels of inflammatory markers TNF-α, GM-CSF, IL-1β and IL-6R tended to be associated with an increased chance of PTD, these associations were not statistically significant (Taylor et al., 2013). In the recent systematic review performed by Wei et al., IL-6 in cervico-vaginal fluid and IL-6 and C-reactive protein in amniotic fluid, but not in plasma, were strongly associated with spontaneous PTD in asymptomatic women (Wei et al., 2010). Furthermore, in their meta-analysis, there was no evidence that other inflammatory cytokines (IL-1, IL-2, IL-8, IL-10 and TNF-α) were associated with spontaneous preterm birth in asymptomatic women. Therefore, among the various inflammatory cytokines reported, targeting IL-6 signaling appears to be the most reasonable for the prevention of PTD in humans.

In the present study, we examined the expression pattern of IL-6 in CAM-complicated placentae and found that macrophages confirmed by CD-68-positive staining was seen in (A), while vimentin staining was negative in (C). (D) Negative control. Bar represents 25 μm. (E) RT-PCR of IL-6, IL-6R, sIL-6R and gp130 from hAECs. SKOV3ip1, a human ovarian cancer cell line, was loaded as a positive control. hAECs expressed neither IL-6 nor IL-6R, while gp130 was expressed. PCR conditions are described in Materials and Methods. M, marker; A, hAECs; S, SKOV3ip1. Blots are representative of three experiments. (F) Western blotting. hAECs were stimulated with 100 ng/ml IL-6 and 60 ng/ml soluble IL-6 receptor (sIL-6R) for 24 h in the presence of control IgG (Control) or 10 μg/ml tocilizumab (TCZ). Cell lysates were collected and resolved by SDS–PAGE and immunoblotted with an antibody against COX-2. β-Actin was used as a loading control. (G) ELISA. PGE2 concentration in culture media from hAECs stimulated with 100 ng/ml IL-6 and 60 ng/ml soluble IL-6 receptor (sIL-6R) for 24 h in the presence of control IgG or 10 μg/ml tocilizumab. *P < 0.05, **P < 0.01, n.s, not significant.
Previous in vitro studies have shown that the resident cells of the chorion and decidua are capable of synthesizing and secreting IL-6, and that this production is stimulated by bacterial LPS or proinflammatory cytokines such as IL-1β and TNF-α (Dudley et al., 1992; Keelan et al., 1997). However, it is also well known from studies of human tissues that IL-6 production is up-regulated in fetal membranes and decidual tissue at term in normal pregnancy, suggesting that IL-6 might be a key regulator acting in late gestation to accelerate the events of labor (Robertson et al., 2010). Recently, Lockwood et al. suggested that decidual cells are a potential source of the characteristic elevation of IL-6 expression observed in patients complicated with pre-eclampsia (Lockwood et al., 2008) or with CAM (Lockwood et al., 2010). Although we stained residual decidual tissues from CAM-complicated placentae as well as the idiopathic preterm control and compared the expressional patterns, neither displayed positive IL-6 immunoreactivities in decidua and we failed to observe significant differences, unlike placental villi. Further research would be required to conclude what types of cells are truly responsible for IL-6 production in patients complicated with CAM.

In our LPS-induced PTD mouse model, 1 mg/kg MR16-1 was administered intraperitoneally 24 h before LPS treatment; however, the finding that IL-6 in cervico-vaginal fluid, but not in plasma, was strongly associated with spontaneous PTD suggested that inflammation at the maternal-fetal interface, rather than systemic inflammation, may play a major role in the etiology of PTD (Wei et al., 2010). In particular, tocilizumab has a molecular weight of 148 000 and does not appear to reach the mice and their pups. In hAECs, pretreatment with tocilizumab significantly inhibited the production of prostaglandin E2 induced by IL-6. We believe that targeting the IL-6 pathway, such as tocilizumab, might be worth evaluating as a novel therapeutic agent for preventing preterm birth.

Supplementary data
Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors’ roles
A.W. performed experiments; K.S. designed the study and wrote the manuscript; M.N. performed experiments; S.M. performed experiments; A.T. performed experiments; Y.K. performed experiments; K.N. performed experiments; K.T. supervised experiments; H.K. supervised experiments; T.K. supervised experiments. All authors approved the submitted version of the manuscript.

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Conflict of interest
None declared.

References


