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J Immunol 2012; 188:5706-5712; Prepublished online 27 April 2012; doi: 10.4049/jimmunol.1103454
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TLR9 Provokes Inflammation in Response to Fetal DNA: Mechanism for Fetal Loss in Preterm Birth and Preeclampsia


Preterm birth, the major cause of neonatal mortality in developed countries, is associated with intrauterine infections and inflammation, although the exact mechanisms underlying this event are unclear. In this study, we show that circulating fetal DNA, which is elevated in pregnancies complicated by preterm labor or preeclampsia, triggers an inflammatory reaction that results in spontaneous preterm birth. Fetal DNA activates NF-κB, shown by IκBα degradation in human PBMCs resulting in production of proinflammatory IL-6. We show that fetal resorption and preterm birth are rapidly induced in mice after i.p. injection of CpG or fetal DNA (300 pg/dam) on gestational day 10–14. In contrast, TLR9−/− mice were protected from these effects. Furthermore, this effect was blocked by oral administration of the TLR9 inhibitor chloroquine. Our data therefore provide a novel mechanism for preterm birth and preeclampsia, highlighting TLR9 as a potential therapeutic target for these common disorders of pregnancy. The Journal of Immunology, 2012, 188: 5706–5712.

Preterm birth (PTB), defined as delivery before 37 wk gestation, has become an epidemic in developed countries. Indeed, PTB and preeclampsia (PEC) are leading causes of maternal and neonatal death worldwide. Premature infants who survive are inflicted with many lifelong handicaps including cerebral palsy and mental retardation (1). The most significant risk factor for PTB is a prior preterm delivery, although both PTB and PEC have been associated with multiple other factors including infection and inflammation (2). Indeed, the innate immune system has been shown to play a role during pregnancy and immunogenic disorders of pregnancy. TLRs are an important class of pathogen recognition receptors, which signal during infection to bring about immune reactions that have been implicated in PTB (3). TLR2 and TLR4, which signal in response to bacterial lipoproteins and LPS, respectively, are highly expressed in the amniotic tissues of women who delivered prematurely, with increased expression of TLR2 and TLR4 in the chorioamniotic membranes (4–8). The Asp299Gly polymorphism, a variant of the human TLR4 gene, has been associated with PTB (8). However, despite these associations, it remains unclear what exact role microbial infection has in PEC as microbial products are rarely detected in the maternal circulation, and furthermore it has been estimated that only 30% of PTB cases demonstrate evidence of microbial infection (9). To further this discrepancy, antibiotic treatment has never been shown to decrease rates of preterm delivery (10). In one study, antibiotic treatment of at-risk patients caused delivery earlier than that for patients not treated with antibiotics, highlighting a lack of knowledge of the exact mechanism underlying PTB and PEC (11–13). Although bacterial and viral factors may incite the inflammatory process without evidence of infection by cultures or histology, it is possible that another more sterile process might be at work. Notably, samples of amniotic fluid and placenta were examined for bacterial products at elective section of term pregnancies, and 70% had evidence of the presence of microbial products by fluorescence in situ hybridization without infection or symptoms of labor or PEC (14). This presents an incongruity with respect to factors from an infective source being the sole cause of inflammatory disorders of pregnancy even at a local level. The concept that, as fetal cells die from whatever insult (subclinical infection, growth restriction, trauma), they release hypomethylated fetal DNA fragments that function as a “danger signal” from the fetus to the mother in order to start an inflammatory process to begin labor would be a unifying mechanism for many of the risk factors for PTB.

A possible clue as to the cause of PTB and PEC is the observation that cell-free fetal DNA can be found in maternal plasma, and furthermore circulating levels of free fetal DNA are significantly elevated in complicated pregnancies (15). This source of free fetal DNA provides a putative danger signal to the maternal immune system, which can induce a proinflammatory response. This process has been termed “danger signal,” and the receptor for this signal is TLR9. TLR9 is expressed in a variety of immune cells, and its activation by certain nucleic acids can lead to the release of proinflammatory cytokines (16–18). Fetal DNA, which is present in the maternal circulation, is a potential danger signal for the maternal immune system. In this study, we show that fetal DNA activates NF-κB, shown by IκBα degradation in human PBMCs resulting in production of proinflammatory IL-6. We show that fetal resorption and preterm birth are rapidly induced in mice after i.p. injection of CpG or fetal DNA (300 pg/dam) on gestational day 10–14. In contrast, TLR9−/− mice were protected from these effects. Furthermore, this effect was blocked by oral administration of the TLR9 inhibitor chloroquine. Our data therefore provide a novel mechanism for preterm birth and preeclampsia, highlighting TLR9 as a potential therapeutic target for these common disorders of pregnancy.
DNA is likely to arise from either destruction by the maternal immune system of fetal cells, which occasionally cross the placental barrier, or from placent al and fetal cells that undergo apoptosis (16). Although normal pregnancies have a certain amount of fetal DNA present within the maternal circulation, this is quickly cleared after delivery (17). However, many different complications of pregnancy are associated with an increased amount of fetal DNA being found in the maternal circulation, with PE and PTB having free fetal DNA concentrations between 2 and 10 ng/ml or 3 µg/ml or 3 µg/ml. Fetal DNA was cleaved by HPAII (Fig. 1B, lane 1) and this cleavage was prevented by inhibition of TLR9 with the TLR9 inhibitor chloroquine, highlighting a potential clinical application. Our work therefore provides a novel mechanism for fetal loss in PTB and PEC and suggests that targeting of TLR9 could have clinical utility.

Materials and Methods

Cell culture

PBMCs isolated from female donors (both nonpregnant and pregnant) and Namalwa cells were cultured in DMEM-glutamax (Life Technologies) supplemented with 10% FCS (Sigma) and 1% penicillin–streptomycin (Sigma) (DMEM–FCS–PS) and seeded at 2.5 × 10^6 cells/ml for experimental assays. Primary bone marrow-derived macrophages (BMDMs) were generated from bone marrow isolated from wild-type mice and cultured in DMEM–FCS–PS supplemented with M-CSF (20% L929 cell supernatant) for 10 d and seeded at 5 × 10^5 cells/ml for assay.

IkBa degradation

Cells were stimulated for various times with either fetal DNA (22-wk female fetus; Biochain), adult DNA (blood of a 50-y-old female; Biochain) or human CpG (Invigene) at 1.5 µg/ml or 3 µg/ml. IkBa degradation was measured by immunoblot analysis using an anti-IkBa Ab (gift from Prof. R. Hay, University of Dundee, Dundee, U.K.). The mAb used to detect β-actin was from Sigma.

ELISA analysis

For animal studies, Student t test was performed to test statistical significance at p < 0.05; data are expressed as means ± SD. For animal studies, one-way ANOVA analysis was performed to test statistical significance at p < 0.05.

Results

Hypomethylated fetal DNA triggers inflammation in human B cells and PBMCs

Initially, we tested a model cell line, Namalwa, a human B cell line that expresses high levels of TLR9. Fetal DNA activated the proinflammatory transcription factor NF-kB as evidenced by IkBa degradation at 5 min stimulation (Fig. 1A, top panel), whereas CpG DNA was active at 15 min (Fig. 1A, middle panel). Adult DNA, which is not hypomethylated, had no effect (Fig. 1A, bottom panel). We next tested the effect of fetal DNA in a more relevant cell population, PBMCs prepared from female donors (who were not pregnant but were of child-bearing age). Both fetal DNA and CpG induced IkBa degradation at 5 min of stimulation (Fig. 1B, top and middle panels, respectively). Adult DNA did not induce IkBa degradation (Fig. 1B, bottom panel). Both fetal DNA and CpG induced IL-6 production in PBMCs up to 4-fold over control (Fig. 1C). Furthermore, fetal DNA significantly induced IL-6 production in PBMCs isolated from pregnant women (n = 5, average donor age 32.6 y, average gestational age 25 wk, two donors had no prior pregnancy, three donors had one prior pregnancy) (Fig. 1D). Fetal DNA did not, however, induce IL-6 in primary murine BMDMs, despite CpG being capable of inducing an IL-6 response in this cell type (Fig. 1E). LPS, which was included as a control, also induced IL-6 in these cells (Fig. 1E). We next analyzed the methylation status of fetal DNA by performing a restriction digest with HPAII, an enzyme that cleaves DNA at unmethylated CpG islands. Fetal DNA was cleaved by HPAII (Fig. 1F, compare lane 2 with lane 1), and this cleavage was prevented by pretreatment with CpG methyltransferase and S-adenosyl methionine CpG methyltransferase enzyme and visualized by gel electrophoresis.

Animals and treatment

Male and female specific pathogen-free BALB/c mice (obtained from Harlan UK) or C57Bl/6 and C57Bl/6 TLR9-ko mice (a gift from K. Fitzgerald, University of Massachusetts) were obtained at 6–12 wk of age. Males were housed individually, and females were housed in groups of five per cage. Food and water were supplied ad libitum. Upon commencement of breeding, one male was added per two females, and females were examined for presence of vaginal plugs (evidence of copulation) the following morning. The morning that the plug was found was considered day 1 of gestation. Pregnant mice were administered PBS (vehicle), LPS (300 µg/dam), CpG ODN (300 µg/dam), fetal DNA (300 µg/dam), or adult DNA (300 µg/dam) by i.p. between gestation days 10 and 14. To determine if chloroquine could be used to prevent fetuin resorption, chloroquine (CLQ; 1 mg/dam) was administered orally at 3 h prior to injection with fetal DNA followed by an additional 2 mg/dam CLQ administered orally 3 h after injection with fetal DNA. Forty-eight hours after injection of fetal DNA, pregnant dams were sacrificed via CO2 inhalation, terminal blood was collected, and serum stored at −80°C for cytokine analysis. The gravid uterus were removed and weighed and the number of resorbed fetuses determined. Placentas and fetuses were preserved in 10% formalin until histological analysis. All animal procedures were approved by the Ethical Review Board, Trinity College Dublin, in accordance with the Cruelty to Animals Act 1876.

Immunohistochemistry

Mouse gestational sacs were removed from uterine horns and placed in 10% buffered formalin and paraffin embedded 24 h after fixation. Samples were stained with H&E. IL-6 and TNF-α were detected using a rabbit-specific HRP/3,3′-diaminobenzidine detection immunohistochemistry kit (Abcam).
Fetal DNA triggers inflammation in Namalwa B cells and human PBMCs. (A) IκBα degradation in Namalwa cells stimulated with fetal DNA, CpG, and adult DNA. (B) IκBα degradation in PBMCs stimulated with fetal DNA, adult DNA, and CpG DNA. (A and B) are representative of three independent experiments. (C) ELISA analysis of IL-6 response in PBMCs from female donors who were not pregnant, stimulated with fetal DNA and CpG. Values are means ± SD, n = 3, *p < 0.05 (representative of three nonpregnant female donors). (D) ELISA analysis of IL-6 response to fetal DNA in PBMCs from pregnant donors. Values are means ± SD, n = 3, *p < 0.05 (representative of five pregnant donors). (E) ELISA analysis of IL-6 response in primary BMDMs stimulated with fetal DNA, CpG, and LPS. Values are means ± SD, n = 3, *p < 0.05 (representative of three independent experiments). (F) Fetal DNA contains hypomethylated CpG as shown by HPAII restriction enzyme digestion of nonmethylated DNA (lane 3) compared with fetal DNA pretreated with CpG methyltransferase and S-adenosyl methionine (lane 4) and adult DNA (lane 6) for 24 h. Control fetal DNA (lane 2) and adult DNA (lane 5) were not exposed to the restriction enzyme. Representative of three independent experiments.

Thus, we included both LPS and CpG as positive controls in our model in which BALB/c dams were administered i.p. either PBS, LPS (300 μg/dam), CpG (300 μg/dam), or fetal DNA (300 μg/dam) between days 10 and 14 of gestation (Fig. 2). We observed 100% (n = 14 of 14) and 72% (n = 26 of 36) resorption after administration of LPS and CpG, respectively (Fig. 2A). Importantly, administration of fetal DNA induced 61% (n = 33 of 54) resorption (Fig. 2A). Negligible resorption was observed in dams administered PBS (n = 1 of 44). As the fetal DNA that we used was of human origin, we also included human adult DNA as a control. Indeed, administration of adult DNA at 300 μg/dam did not induce a significant amount of resorption (Fig. 2A). Strikingly, H&E staining of the placenta after maternal treatment during gestation with CpG or fetal DNA demonstrated significant infiltration of the placental site by neutrophils and monocytes as well as separation of the placental site from the uterine wall, which was accompanied by degradation and buildup of cellular debris (Fig. 2B, micrographs 3 and 4, respectively). In micrograph 3, an infiltrate of monocytes and polymorphonuclear cells (PMNs) are noted accompanied by separation of the trophoblastic tissue (giant cell trophoblast; GCT) from the uterine muscle (UM) after administration of CpG. In micrograph 4, clumps of PMNs (small ring) and monocytes (large ring) are indicated along with separation of GCT from the UM (indicated by arrow). In contrast, the placental sites of mice injected with PBS or adult DNA were without significant infiltrates and remained intact (Fig 2B, micrographs 1 and 2, respectively). In micrographs 1 and 2, GCTs of the placenta are attached to UM, and occasional PMNs are seen within the maternal blood vessel in a singular arrangement (25). Notably, whereas CpG induced systemic production of a range of cytokines (IFN-γ, IL-12p70, mKC, TNF-α, IL-10) in mice, fetal DNA did not induce this response suggesting a localized effect (data not shown). To confirm whether this was indeed a localized effect, immunohistochemistry was performed on tissue sections for the presence of TNF-α (Fig. 2C) and IL-6 (Fig. 2D), two cytokines that have previously been implicated in inflammatory disorders of pregnancy (26). Dams treated with PBS and adult DNA demonstrated no evidence of TNF-α in the endometrium (Fig. 2C, micrographs 1 and 2, respectively), whereas dams administered CpG and fetal DNA showed positive immunohistological staining for TNF-α (Fig. 2C, micrographs 3 and 4, respectively). Similarly, there was a lack of IL-6 staining in dams administered PBS and adult DNA (Fig. 2D, micrographs 1 and 2, respectively) compared with dams administered CpG DNA (Fig. 2D, micrograph 3) and fetal DNA (Fig. 2D, micrograph 4). Infiltration of inflammatory cells was observed in sections from mice administered CpG and fetal DNA (Fig. 2E, first column, middle and bottom panels). Colocalization of PMNs and monocytes with the inflammatory cytokines is indicated by circles (Fig. 2E, middle and right-hand columns, middle and bottom panels).

FIGURE 1. Fetal DNA triggers inflammation during pregnancy.
Fetal DNA is sensed by TLR9 to induce fetal resorption

Having already demonstrated that fetal DNA could activate NF-κB and induce IL-6 in vitro and also induce fetal loss and cytotrophoblastic inflammation in vivo in mice, we next tested whether its effect was TLR9 dependent. As we had previously seen that hypomethylated fetal DNA activated Namalwa cells, which express high levels of TLR9, we sought to determine whether fetal DNA could be sensed by TLR9. Thus, using our model of PTB, we compared fetal resorption in wild-type C57bl/6 and C57bl/6 TLR9-deficient mice (Fig. 3). Fetal DNA induced only 12% (n = 3 of 25) resorption in TLR9-deficient mice compared with 85% (n = 29 of 34) resorption in wild-type mice (Fig. 3A). The resulting fetal resorption correlated with a significant reduction in fetal weight in wild-type mice (Fig. 3B). H&E staining of the placenta from wild-type and TLR9-deficient mice subjected to immunohistochemistry for TNF-α and IL-6, as indicated by brown staining. Original magnification ×400. (E) H&E staining of the placenta after maternal treatment during gestation with adult DNA (top row), CpG (middle row), or fetal DNA (bottom row) demonstrating the presence of TNF-α or IL-6 protein within the cytoplasm of neutrophils and monocytes (25). Original magnification ×400.

CLQ prevents fetal DNA-induced fetal loss in mice

Next, we investigated the potential therapeutic effect of the TLR9 inhibitor CLQ in our model. CLQ has been shown to inhibit TLR9 by blocking acidification of the endosome, which is required for TLR9 signaling (27). First, we observed that pretreatment of Namalwa cells with CLQ or an inhibitory ODN to TLR9 diminished fetal DNA-induced NF-κB degradation in these cells compared with untreated cells stimulated with fetal DNA (compare bottom panel with top panel in micrograph 6). Furthermore, the placental sites of wild-type mice were laden with calcifications indicative of fetal loss and a dead resorbing placenta (Fig. 3C, micrograph 4, indicated by arrow). In contrast, inflammatory TNF-α (micrograph 3) and IL-6 (micrograph 5) were notably absent in the placenta of TLR9-deficient mice, and there was no cellular infiltrate. Macroscopic analysis of uterine horns harvested on gestational day 14 from wild-type and TLR9-deficient mice treated on gestational day 12 with fetal DNA revealed a striking difference in size, indicative of fetal loss in wild-type mice (Fig. 3D). In contrast, TLR9-deficient mice had normal gestational sacs containing normal healthy fetuses demonstrating fetal DNA-induced fetal resorption is TLR9 dependent (Fig. 3D). Thus, our data show that fetal DNA provokes fetal loss via TLR9 activation within the uteri of mice.
FIGURE 3. Fetal DNA is sensed by TLR9 to induce fetal resorption. (A) TLR9-deficient mice are resistant to fetal DNA as shown by measurement of fetal resorption in pregnant C57bl/6 wild-type and C57bl/6 TLR9-deficient mice administered i.p. fetal DNA on gestation day 10–14 and sacrificed 48 h later. n indicates number of resorbed fetuses out of total number of fetuses. *p < 0.05 (significantly different from control group). (B) TLR9-deficient mice are resistant to resorption induced by fetal DNA as shown by fetal weights. (C) H&E staining of the placenta from C57bl/6 wild-type and TLR9-deficient mice after maternal treatment during gestation with fetal DNA (1 and 2). Arrow indicates calcification. Sections were stained for the presence of TNF (3 and 4) or IL-6 (5 and 6), indicated by presence of brown staining. Original magnification ×400. (D) Representative uterine horns harvested on gestational day 14 from C57bl/6 wild-type and TLR9-deficient mice treated on gestational day 12 with fetal DNA demonstrating fetal DNA-induced fetal resorption is TLR9 dependent.

Discussion

Despite advances in the management of high-risk pregnancies, such as those complicated by PTB and PEC, the incidence of these complications remains high, with knowledge of the underlying mechanisms still unclear. However, it has become obvious that there is an intimate relationship between the maternal immune system, the fetus, and their relative environments, and recent studies have begun to investigate more closely this relationship. In this study, we have analyzed the proinflammatory potential of human fetal DNA, which is found at increased concentrations in the circulation of pregnant women undergoing PTB or PEC. Fetal DNA caused activation of the transcription factor NF-kB, leading to the production of proinflammatory IL-6. Furthermore, using a mouse model, we investigated the consequences of fetal DNA administration in pregnant BALB/c and C57bl/6 mice. We observed that when injected i.p. on day 10–14 of gestation, fetal DNA caused rapid fetal resorption in both BALB/c and C57bl/6 mice and a localized inflammatory response in the uterus, as shown by infiltration of PMNs and monocytes and cytokine production by these cells. This inflammatory effect of fetal DNA is dependent on TLR9, as C57bl/6 TLR9-deficient mice experienced significantly reduced fetal resorption in response to fetal DNA. Furthermore, oral administration of the TLR9 inhibitor CLQ significantly protected BALB/c mice from fetal resorption.

Our data provide a new model for PTB, a recently reported deficiency in this field, highlighted by a call by Nature Medicine for better models to study this clinical dilemma, with hopes that research resulting from these models would lead to an increased understanding of PTB, ultimately leading to a decreased incidence and improved neonatal outcome. Our study lends itself to this cause. CLQ has been given to pregnant mothers with malaria and systemic lupus erythematosus for decades. CLQ crosses the placenta as its metabolites are found in the cord blood, systemic blood, and urine of neonates delivered from mothers treated with CLQ. As TLR9 responsive cells are maternal monocytes, dendritic cells, NK cells, macrophages, and trophoblasts of the human placenta, it is likely that such a therapy would reach the local cells likely to be involved. Our observations highlight a further therapeutic use for CLQ during pregnancy.

In our study, we observed infiltration of leukocytes into the murine placenta after administration of fetal DNA. This inflammatory cell infiltration was notably absent in mice administered
either PBS or adult DNA and in TLR9-deficient and CLQ-treated mice. This inflammatory infiltration correlates with observations in women of increased infiltration of neutrophils into the uterine muscle after the onset of labor (30). Furthermore, we observed localized staining of proinflammatory IL-6 and TNF-α in placental tissues from mice administered fetal DNA. Again, this was absent in mice administered either PBS or adult DNA and in TLR9-deficient and CLQ-treated mice. IL-6 has previously been associated with PTB, and the concentration of IL-6 present in amniotic fluid is a sensitive test for antenatal diagnosis and can predict perinatal morbidity (31, 32). Absence of a proinflammatory profile in the systemic circulation of mice administered fetal DNA suggests a localized effect. This is further supported by a lack of activation of BMDMs while fetal DNA did not activate both a Namalwa B cell line and PBMCs from both pregnant and nonpregnant donors, again suggesting that the target cell is localized to the uterus, perhaps being either a monocyte or NK cells. Indeed, TLR9 is expressed by numerous cells of the immune system including NK cells and monocytes, with its highest expression on dendritic cells and B lymphocytes, and reduced expression on monocyte-derived macrophages (33–39). Furthermore, why BMDMs did not respond to fetal DNA but did respond to CpG DNA is not clear but could be due to macrophages being less responsive overall to TLR9 stimulation.

Increased concentrations of fetal DNA present in the maternal circulation is therefore a likely trigger for inflammation that results in complications of pregnancy including PTB, PEC, growth retardation, and stillbirth. High levels of free fetal DNA at 11–14 wk gestation are predictive of poor fetal outcome including delivery before 35 wk gestation and increased likelihood of growth restriction or PEC (40). Pregnant women who experience one of the most severe forms of PEC, HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count) have the highest levels of cell-free fetal DNA in the maternal circulation, nearly 10-fold higher than those in normal pregnancies at the same gestational age (41). High levels of fetal DNA are also detected in the maternal circulation during placental abruption, which results in delivery, disseminated intravascular coagulation, and often fetal death (42). It is likely that high levels of free fetal DNA in maternal circulation functions as a danger signal to the mother that fetal cells are dying, leading to inflammation and delivery or death of the fetus. One must also consider that genetic variation in TLR9 may predispose women to fetal loss. We are currently examining this possibility.

Our observation raises the possibility that inhibition of TLR9 through administration of CLQ or ODNs that antagonize TLR9 to mothers with high levels of fetal DNA detected in their circulation might prevent two of the most common and detrimental disorders of pregnancy.
Disclosures
The authors have no financial conflicts of interest.

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