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Increased TLR4 Expression in Murine Placentas after Oral Infection with Periodontal Pathogens

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ABSTRACT

Maternal periodontitis has emerged as a putative risk factor for preterm births in humans. The periodontitis-associated dental biofilm is thought to serve as an important source of oral bacteria and related virulence factors that hematogenously disseminate and affect the fetoplacental unit; however the underlying biological mechanisms are yet to be fully elucidated. This study hypothesized that an oral infection with the human periodontal pathogens *Campylobacter rectus* and *Porphyromonas gingivalis* is able to induce fetal growth restriction, placental inflammation and enhance Toll-like receptors type 4 (TLR4) expression in a murine pregnancy model. Female Balb/C mice ($n = 40$) were orally infected with *C. rectus* and/or *P. gingivalis* over a 16-week period and mated once/week. Pregnant mice were sacrificed at embryonic day (E) 16.5 and placentas were collected and analyzed for TLR4 mRNA levels and qualitative protein expression by real-time PCR and immunofluorescence. TLR4 mRNA expression was found to be increased in the *C. rectus*-infected group (1.98 ± 0.886-fold difference, $P < 0.01$, ANOVA) compared to controls. Microscopic analysis of murine placentas showed enhanced immunofluorescence of TLR4 in trophoblasts, mainly in the placental labyrinth layer. Also, combined oral infection with *C. rectus* and *P. gingivalis* significantly reduced the overall fecundity compared to controls (16.7% vs. 75%, infected vs. non-infected mice respectively, $P = 0.03$, Kaplan–Meier). The results supported an enhanced placental TLR4 expression after oral infection with periodontal pathogens. The TLR4 pathway has been implicated in the pathogenesis of preterm births; therefore the abnormal regulation of placental TLR4 may give new insights into how maternal periodontitis and periodontal pathogens might be linked to placental inflammation and preterm birth pathogenesis.

1. Introduction

Preterm birth (birth at ≤37 completed gestational weeks) is the major cause of human neonatal mortality/morbidity in the world, accounting for up to 75–85% of the early neonatal deaths as well as high rates of short-term (low birth weight) and long-term adverse sequelae (hearing/learning problems and cerebral palsy) [1,2]. The pathogenesis of preterm birth is thought to be multi-factorial, possibly initiated by multiple mechanisms including infection, uteroplacental ischemia, hemorrhage, stress and other immunologically mediated processes [3]; however, the development of a pro-inflammatory condition is a common effector pathway that centralizes all multiple risk factors [4]. In particular, uterine infections may account for 25–40% of preterm births and they are strongly linked with a pro-inflammatory systemic state. For example, uterine infections are known to upregulate the production of local pro-inflammatory cytokines, metalloproteinases and prostaglandins that lead to membrane weakening, early membrane rupture and uterine contraction initiation [5]. Uterine infections usually take advantage of ascending mechanisms, which are originated from vaginal infections (e.g., *Neisseria gonorrhoeae* or *Ureaplasma urealyticum*) that lead to intrauterine cavity access, decidua colonization, localized inflammation onset (or chorioamnionitis), intraamniotic infection and, ultimately, fetal infection [6].

Nonetheless, other sources of infection including the oral cavity have been proposed to facilitate the hematogenous transmission of pathogens that affect normal pregnancy development [7]. In particular, periodontal diseases (gingivitis and periodontitis) are among the most common chronic infections, affecting up to 50% of humans [8] and have been found to be an independent putative risk factor for pregnancy-related complications such as preterm births,

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low birth weight and preeclampsia, after adjusting for other known obstetric risk factors [9,10]. Periodontitis is initiated when specific microorganisms accumulate between the teeth and the gums, forming bacteria-rich biofilms commonly known as dental plaque. The body reacts to dental biofilms by activating the oral mucosal inflammatory response that – in some susceptible patients – is unsuccessful in controlling the infection. With time, the inflammatory response remains chronic and ultimately leads to periodontal connective tissue resorption (alveolar bone loss) and tooth loss [11]. During prolonged periodontal inflammation, periodontal pathogens and related virulence factors invade periodontal tissues, enter the blood stream in the form of transient bacteremias [12] and disseminate throughout different systemic organs. In fact, important periodontal pathogens have been detected in human placentas of women with preeclampsia [13] and in the amniotic fluid of pregnant women with a diagnosis of premature labor [14] or premature labor with intact membranes [15,16].

Fetal exposure to periodontal pathogens from maternal oral biofilms has also been demonstrated in umbilical cord blood samples from preterm births by detecting maternal immunoglobulin G (IgG) as well as fetal immunoglobulin M (IgM) to one or more specific oral pathogens. In particular, mothers with a low IgG response to *Porphyromonas gingivalis* combined with a high fetal IgM response to *Campylobacter rectus* showed the highest rate of preterm deliveries (66.7%) among 812 deliveries from a cohort study of pregnant mothers (adjusted OR 10.3; $P < 0.0001$) [17], suggesting that *P. gingivalis* and *C. rectus* could act as fetal infectious agents eliciting complications during pregnancy. *C. rectus* is a Gram negative anaerobe, and a motile bacterium unique to the oral cavity, that is phylogenetically related to *Helicobacter pylori* and is associated with ulceration of the periodontal attachment apparatus [18]. Interestingly, other *Campylobacter* species are known to be a significant causal agent of sheep and cattle abortion due to a marked tropism for placental tissues [19]. In animal models, *Campylobacter jejuni* and *Campylobacter fetus* infections result in impaired development and fetal growth restriction (FGR) [20]. We have previously reported that a subcutaneous infection with *C. rectus* in pregnant mice disseminates to placental tissues and induces FGR [21,22], placental inflammation and structural alterations [23]. Likewise, animal experiments using *P. gingivalis* in a subcutaneous infection model have shown increased maternal inflammatory serum markers (interleukin-6 and tumor necrosis factor alpha TNF-α), and increased fetal biochemical markers of placental inflammation (prostaglandin E_2) in murine amniotic fluid [24,25].

Placental infection and subsequent inflammation have been associated with preterm labor, so the biological pathways related to early inflammatory responses are likely to mediate pathogenesis. Toll-like receptors (TLRs) are pattern recognition receptors that play a key role in the innate inflammatory response [26] and have been proposed to play important roles in pregnancy maintenance, placental immune protection and delivery initiation [27]. To date, a total of 10 human and 12 murine TLRs have been described. In general, TLRs can be categorized into two main groups based on their ligands: the first group consists of TLR1, 2, 4, and 6 which recognize bacterial molecules such as lipopolysaccharide (LPS), lipoteichoic acid and peptidoglycan. The second group consists of TLR3, 7, 8, and 9 that recognize pathogen-associated nucleic acid patterns [28]. Here we focused on TLR4, which is selectively activated by Gram negative LPS, in conjunction with CD14 [29]. Since *P. gingivalis* and *C. rectus* are Gram negative periodontal pathogens, the main objective of this study was to determine whether an oral infection with *C. rectus* and *C. rectus*/*P. gingivalis* combined infection could affect fetal growth, or fecundity, and induce placental inflammation along with enhanced expression of TLR4 in a timed-pregnancy murine model.

2. Methods

2.1. Timed-pregnancy murine model

Balb/C mice were obtained at 6–8 weeks of age and maintained on a 12-h light/dark cycle (0700–1900 light) and a constant temperature of 25 °C, receiving distilled water and food *ad libitum*. To facilitate bacterial colonization, all females were changed to a soft chow enriched with a dextrose solution (30%) as a plaque-promoting diet during the infection period. For mating purposes, females were age-matched when 20 weeks old, and males were permanently randomized to specific experimental groups. Female pregnancies were confirmed by the presence of a vaginal plug plus significant weight changes (>1.5 g gain in a week). Mice were infected daily over a 16-week period and mated once/week. When pregnant, female mice were sacrificed at embryonic day (E) 16.5 and placental tissues were collected and analyzed. All procedures were in accordance with animal guidelines approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

2.2. Bacterial strains and inoculum preparation

C. rectus 314 and *P. gingivalis* A7436 aliquots were maintained in Wilkins Chalgren anaerobic broth medium (WC broth; DSMZ, Braunschweig, Germany) containing 10% skim milk at −80 °C. *C. rectus* aliquots were reconstituted on PRAS ETSA plates (Enriched Tryptic Soy Agar from Anaerobe Systems, Morgan Hill CA) and *P. gingivalis* aliquots on Anaerobic Reducible Blood Agar (from Remel, Lenexa, KS). For experiments, bacteria were anaerobically grown under 5% CO_2, 10% H_2–85% N_2 atmosphere at 37 °C for 4–6 days. Bacterial suspensions were prepared from primary cultures at their log phase of growth, and concentrations were determined by spectrophotometry (Cecil Instruments, Cambridge, UK) with a measured optical density at 600 nm (*C. rectus*) and 660 nm (*P. gingivalis*) corresponding to 1×10^9 bacteria/ml respectively. Finally, all oral preparations were adjusted accordingly to keep the same concentration during oral infection experiments.

2.3. Oral infection

The oral infection model of experimental periodontitis has been described elsewhere [30]. This model involves a pretreatment phase with antibiotics to suppress the oral flora to permit the colonization of exogenously applied human oral bacterial strains and the use of carboxymethylcellulose (CMC) as a carrier to facilitate bacterial colonization. Briefly, before experiment baseline 40 female Balb/C mice were pretreated for 4 days with Kanamycin/Ampicillin (50/25 mg/kg) followed by a 3-day antibiotic wash out period, and then randomly assigned to experimental groups (Table 1 and Fig. 1). Mice were topically infected in the oral cavity with 1×10^{10} live bacterial units in 100 μl of a phosphate-buffered saline (PBS) and 2% CMC preparation on a daily basis. Controls included a blank group (same diet without oral infection) and a negative control group (CMC application without bacteria).

2.4. Placental histology and immunofluorescence

Placentas were fixed in 4% paraformaldehyde, bisected sagittally, processed and embedded in paraffin. Sections (~5 μm) were stained using hematoxylin/eosin (H&E) for structural analysis; other sections were processed for immunofluorescence. Briefly, tissue antigens were rescued with Safeclear® (Fisher Protocol, Fair Lawn, NJ) for 20 min and washed under serial ethanol concentrations. After washing in 0.2% Triton/PBS, sections were incubated in 10% non-immune goat serum and bovine serum albumin in PBS for 1 h. Rabbit anti-TLR4 monoclonal antibody (Zymed, Invitrogen, Carlsbad, CA) and mouse anti-mouse Cytokeratin 7 monoclonal antibody (RCK105 from Abcam, Cambridge, UK) were incubated overnight at a 1:50 concentration. Cytokeratin 7 was chosen as a trophoblast marker following the recommendations of the workshop report on cell culture models of trophoblasts [31]. After vigorous washing, secondary biotinylated antibodies were applied for 1 h (Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG from Molecular Probes, Invitrogen, Carlsbad, CA) for 1 h. Sections were washed in 0.2% Triton/PBS, mounted and coverslipped with Vectashield (Vector labs, Burlingame, CA). All stained sections were analyzed and photographed under confocal microscopy (LSM5, Carl Zeiss, Thornwood, NY).

2.5. Quantitative RT-PCR for TLR4

Total RNA was isolated from all placental tissues ($n = 135$) with the use of the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA from 2 μg of total RNA was synthesized using the Omniscript Kit (Qiagen) and random decamer primers. Real-time PCR was performed with 1 μl cDNA, TaqMan Universal PCR mix, and 20X primer (Mm00445273_m1 from Applied Biosystems, Foster City, CA), in a 7000 Sequence Detection System (ABI Prism, Applied Biosystems). Reactions were performed in duplicates and at two independent times. The Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) gene was used as an endogenous control (housekeeping gene). Results were evaluated using the delta–delta Ct method, where delta Ct was calculated as (TLR4 Ct) − (GAPDH Ct), and the relative quantity of TLR4 mRNA expression was calculated by the delta–delta Ct as $2^{-[(\text{infected sample delta Ct})-(\text{control sample delta Ct})]}$.