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Identification of intracellular bacteria in the basal plate of the human placenta in term and preterm gestations

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Abstract

Objective—Bacteria have been identified in different regions of the placenta. Here, we tested the hypothesis that the maternal basal plate of the placenta harbors microbes which may be associated with adverse pregnancy outcomes.

Study Design—We performed a cross-sectional study of pregnancies from a single tertiary care hospital. Maternal medical and obstetric characteristics were obtained and pregnancies followed prospectively for outcomes and placental collection. After delivery, systematic random sampling of the placental basal plate was performed. Paraffin sections of basal plates were stained with four histological stains and scored for morphological evidence of bacteria.

Results—Of 195 total patients in the study, Gram positive and negative intracellular bacteria of diverse morphologies were documented in the basal plates of 27% of all placentas. 35% of the patients delivered preterm. No difference was noted between placental basal plates from preterm or term gestations. Intracellular bacteria were found in the placental basal plates of 54% spontaneous preterm deliveries before 28 weeks, and in 26% of term spontaneous deliveries ($p=0.02$). Intracellular bacteria were also documented in placentas without clinical or pathologic chorioamnionitis.

Conclusions—27% of placentas demonstrated intracellular bacteria in the placental basal plate using morphological techniques. Thus, the maternal basal plate is a possible source of intrauterine colonization and placental pathological examination could include examination for bacteria in this important maternal fetal interface.

Keywords

Infection; Preterm birth; chorioamnionitis

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Introduction

Infection and inflammation are commonly associated with the risk for preterm birth (PTB). Predisposing factors for infection-related preterm delivery are diverse, including subclinical intrauterine infections¹, intra-amniotic infections^{2,3} and pyelonephritis⁴. In addition, maternal history of prior PTB, especially multiple and/or early PTB(s) is a significant risk factor⁵. This recurrence risk suggests the presence of a risk factor that is present from pregnancy to pregnancy. The specific mechanisms by which infectious insults trigger preterm parturition are poorly understood. Notably, randomized trials of antimicrobials for treatment of infection for prevention of PTB have been disappointing⁶⁻⁸.

Previous studies have shown that pathogenic microbes can establish occult intracellular reservoirs within the epithelium of the murine urinary tract, thereby evading immune recognition and allowing re-emergence of symptomatic infection⁹. In addition, prior studies have documented the presence of bacteria in various placental locations¹⁰⁻¹² but have not examined the basal plate specifically. We hypothesized that the cells in the basal plate of the placenta which comprise the tissue layer directly at the maternal fetal interface may harbor occult microbes similar to the findings in previous placental studies as well as our studies in the murine urinary tract. We reasoned that occult microorganisms in the basal plate could lead to chronic or acute inflammation, and may be associated with adverse pregnancy outcomes such as preterm birth and chorioamnionitis. The objective of this study is first, to examine the basal plate of a large group of placentas to diagnose the presence or absence of microbes at the maternal fetal interface. Second, when microbes were identified in the basal plate maternal fetal interface, we sought to investigate whether the presence of bacteria was associated with important clinical outcomes such as chorioamnionitis and preterm birth.

Materials and Methods

Study Design

This is a cross-sectional study of women from a single tertiary care hospital. The study was approved by the IRB of Washington University School of Medicine in St. Louis, MO. Women were enrolled during their antenatal course and followed until delivery. Clinical data and placental specimens were collected through an institutional core resource, the Women's and Infant's Health Specimen Consortium (WIHSC). Gestational age was assigned by the best data available from the last menstrual period (LMP) if consistent with ultrasound dating (± 5 days in the first trimester or ± 14 days in the second trimester). If LMP was unknown, or inconsistent with ultrasound dating, gestational age was assigned according to the earliest ultrasound available. Exclusion criteria were hepatitis B, hepatitis C, and HIV infection. In-depth chart reviews were performed by trained research personnel as the patient progressed through her antenatal and delivery course using close-ended data collection forms. This included maternal medical history, obstetric history, pregnancy diagnoses and outcomes as documented by the treating obstetric team.

Tissue harvest

Placentas (n= 195) were collected 12 h from delivery. Trained research assistants used sterile technique to harvest three random, 5–8 mm samples from the most superficial area of the basal plate equidistant from each other and without inclusion of the periphery of the placental disc^{13, 14}. Specimens were fixed for 48 h at room temperature in 10% neutral buffered formalin prior to embedding in paraffin, as described⁹.

Histopathology and Histochemical analyses

Five micron sections of embedded tissues were deparaffinized with xylene and ethanol and stained with hematoxylin and eosin (H&E) and evaluated for histological evidence of basal plate components including stromal decidual cells and fetal epithelial trophoblasts. Since H&E stain is not sufficiently sensitive to detect bacteria (typically $1 \mu\text{m}$), slides were stained for bacterial detection by three methods: Gram Stain (Acros Organics, Fair Lawn, NJ); Hema 3 modification of the Geimsa stain (Fisher Scientific, St. Louis, MO); and the Brown-Hopps modification of the Gram stain (Fisher Scientific, St. Louis, MO). Ten random fields of each specimen per $100 \mu\text{m}^2$ were examined for the presence or absence of bacteria by two independent observers blinded to clinical history. The histological location of microorganisms was recorded by use of a Nikon Eclipse microscope equipped with an Olympus DP71 color camera.

Exploration of Obstetric Outcomes and Statistical Analysis

Chorioamnionitis and preterm birth were two clinical outcomes of interest in this study. A cohort analysis was performed to explore the association between these two clinical outcomes and bacterial colonization at the maternal fetal interface. Pregnancies with evidence of intracellular bacteria at the basal plate were compared to those without bacteria at the basal plate. PTB was defined broadly as delivery for any reason at less than 37 weeks of gestation. Spontaneous PTB sourced to preterm labor or preterm rupture of membranes as well as indicated PTB due to preeclampsia, abruptio placentae, or intrauterine growth restriction were included¹⁵⁻¹⁷. An analysis of spontaneous preterm birth alone (defined as preterm labor and preterm premature rupture of membranes) was performed. Chorioamnionitis was assigned based on the hospital derived placental pathology reports indicating the presence or absence of acute maternal or fetal inflammation or when antibiotics were administered in labor according to the institutional protocol for presumed chorioamnionitis.

GBS colonization was classified as positive if a vaginal/rectal swab was positive within 5 weeks of delivery, or if a urine culture grew GBS at anytime during the pregnancy. Per routine practice at our institution, all preterm patients receive antibiotic prophylaxis for neonatal Group B Streptococcus (GBS) colonization. Therefore, all women, independent of GBS carriage or treatment status were included in the main analysis. A secondary analysis was performed excluding women with GBS vaginal colonization or urinary infection.

Advanced maternal age was defined as maternal age at delivery ≥ 35 years old. Race, tobacco and alcohol use were based on self-report. Sexually transmitted infections were captured as “during pregnancy” (which included presence of infection at the time of delivery or any time during the current gestation) or “prior to pregnancy” (defined as anytime in the past but predating the current pregnancy). STIs included gonorrhea, chlamydia, genital herpes simplex, trichomonas, and syphilis. Urinary tract infection was assigned if a positive microbial culture was present, or if clinical suspicion was sufficient to treat the patient with antibiotics. All study related clinical information was entered into a secure database using Illume (DatStat, Inc, Seattle WA). Official pathology reports and diagnoses were also reviewed and added to the database.

Demographic information of women with and without intracellular bacteria was compared by t-test, chi-square or Fisher’s exact test as appropriate. Subsequently, univariate analysis was used to compare the incidence of intracellular bacteria in basal plate specimens from term compared to preterm gestations as well as from those with and without the diagnosis of chorioamnionitis. To compare each gestational age group to the reference group of term pregnancies, Poisson regression with robust error was used to estimate the relative risk and

95% confidence intervals. This analytic approach provides an unbiased estimate of the RR when the outcome is common (greater than 10%)¹⁸.

Results

Of 195 pregnancies evaluated in this study, 27% had evidence of intracellular bacteria in the basal plate of the placenta. Demographic characteristics of the patients who participated are demonstrated in Table 1. The multiple histologic stains used in this study assured us that we could determine both morphology and location of bacteria within cells in the basal plate if they were present, as posed in our hypothesis. We identified individual bacteria and biofilm-like clusters of bacteria in the basal plate (Figure 1).

Hema3 modification of the Giemsa stain permits detection of bacteria as well as inflammatory infiltrates. Using this stain, we noted biofilm-like clusters of bacteria stained dark blue/purple in the basal plate (Fig. 1a,b,c). Gram stains were conducted to determine whether the bacteria were Gram Positive or negative. Fig. 1(e, f and g) illustrate examples of Gram positive bacteria. We further found that bacteria of all shape classes were recorded and were of diverse morphologies, including filamentous (Fig. 1e,i), diplococci (Fig. 1f) or streptococci (Fig. 1g), rods (Fig. 1b,c,h), spirochetes (Fig. 1d). The biofilm-like clusters could be both coccoid as in Fig. 1a or rod shaped as in Fig. 1b or 1c. We used the Brown-Hopps stain which detects DNA content, thus rendering the bacteria and host nuclei a deep purple over a pale yellow background of cytosolic components to confirm the presence or absence of bacteria in all the basal plate tissues examined. This stain offers the advantage of rapid and reliable detection of bacteria even at 20× magnification due to the staining patterns. Fig. 1 h and i panels depict bacteria evident in tissue stained with the Brown-Hopps stain.

We examined chorioamnionitis and PTB as two clinical outcomes of specific interest in this study. Thirty-three patients (17%) had either clinical or histologic diagnosis of chorioamnionitis. The finding of intracellular bacteria at the basal plate was no different in patients with or without clinical or histologic diagnosis of chorioamnionitis (Table 2). Sixty-eight patients were delivered preterm and the remainder (n=127) were delivered at term. PTB was no more common in placentas harboring intracellular bacteria at the maternal fetal interface than those without bacteria present (Table 2). As GBS carriage could potentially confound our analysis of intracellular bacteria incidence in basal plate, a secondary analysis was performed excluding women with GBS colonization. The results remained the same with similar rates of intracellular bacteria in preterm vs. term placentas (30.4% vs. 27.4%, $p=0.7$). Among the preterm deliveries, 38.2% (n=26) were delivered between 34–37 weeks' gestation, 39.7% (n=27) between 28–34 weeks' gestation, and 22.1% (n=15) at < 28 weeks' gestation. No significant difference of intracellular bacteria in multiple gestational age strata was noted (Table 3). Fifty of the preterm birth cases (74%) were classified as spontaneous preterm birth. A significant 2-fold increased risk for intracellular bacteria in the basal plates of very preterm placentas (<28 weeks) in the spontaneous preterm birth subgroup was evident when compared to the term birth reference group (54.5% vs. 26.7%, $p=0.02$) (Table 4).

Comment

Principal findings of the study

This study demonstrates the first morphological documentation of intracellular organisms in the decidual basal plate of human placentas. Microbial biofilms, defined as microbial communities encapsulated within polysaccharide matrices¹⁹ have been implicated in >80% of human infections such as periodontitis, urethritis, endocarditis, cystitis, and device-

associated infections. Romero et al. demonstrated that there were biofilms in amniotic fluid^{3,20}. Here, we show that 27% of all placentas harbor intracellular bacteria in the basal plate. These findings underscore our hypothesis that bacteria can colonize the maternal-fetal interface. Our data show that both term and preterm placentas can have intracellular bacteria present without overt infection while a substantial proportion of basal plate specimens even from preterm placentas do not demonstrate bacteria. However, we find a significant increase in incidence of detectable bacteria in the placentas from spontaneous preterm birth at very premature gestational ages. Our findings complement previous studies showing that bacteria are detectable in placentas^{10,11, 12} and recoverable from biopsies of chorion parenchyma from placentas at <28 gestational weeks²¹. Together, these findings are consistent with evidence that presence of microorganisms in placental tissue, whether via culture techniques or morphologically, convey biologically important information.

Clinical Implications

Our novel morphological findings add the maternal basal plate as a hitherto unexplored location for bacteria. The use of high magnification imaging with the use of histologic tissue stains described here to optimize the detection of bacteria may help to better identify the presence of bacteria in the basal plate while also pinpointing their location. The finding of intracellular bacteria of diverse morphologies in the basal plates of more than one-fourth of human placentas in the absence of a diagnosis of chorioamnionitis on the placental pathology report may explain why identification may be easily missed on routine placental pathologic examination and suggests that the basal plate may require more evaluation including special stains for bacteria during routine placental pathological exam. Our findings of the presence of intracellular bacteria, without overt chorioamnionitis or deciduitis, indicates that the microorganisms may, or may not, elicit a host-response that is identifiable as inflammation from the placental histopathology on examination. This is consistent with a recent report that histologic chorioamnionitis may not always indicate presence of an infection and in fact is associated with a reduced risk of sepsis in preterm infants^{22, 23}. Chronic chorioamnionitis is commonly noted in unexplained preterm fetal death²⁴ and may be more representative of maternal antifetal rejection rather than infection²⁵.

Implications of the work for further research

Key findings from our previous studies show that pathogenic microbes can establish asymptomatic occult intracellular reservoirs within the urinary tract epithelium thus evading immune recognition and detection with culture techniques. Specifically, we have elucidated in the urinary epithelium, how uropathogenic *E. coli* bacteria establish occult infections, remain asymptomatic, and yet in the presence of certain stimuli re-emerge and initiate inflammatory cascades causing symptomatic infection⁹. Clinical dogma held that all UTIs, acute or recurrent, are due to bladder colonization by intestinal *E. coli*. However, given that a large percentage of recurrent UTIs are caused by the same strain of bacteria as the initial infection^{26,27,28} and recurrence occurs despite appropriate antibiotic treatment²⁹, we and others established that the bladder itself as a source for bacteria that can seed new infections⁹. Similar to the urinary epithelium, we suggest that the endometrial epithelium of the non-pregnant uterus may harbor occult microbes which become incorporated into the basal plate at the time of placental implantation. Indeed, Andrews and co-workers have shown that micro-organisms can be found in the endometrium^{30, 31}. In the urinary tract, injury to the tissue inducible by infectious or chemical means and consequent tissue remodeling serves as a trigger for re-emergence of the bacteria from their occult niches⁹. Women with a prior PTB are 2–3 times more likely to deliver preterm, and women with 2 prior PTBs have a six-fold increase in risk for another³². Our findings lead us to speculate that conceivably, such occult bacteria in the maternal basal plate could constitute a potential source of intrauterine colonization upon escape from this location^{33, 34}. Our work also

complements previous studies showing that the bacterial pathogen, *L. monocytogenes* can infect fetal epithelial cell cultures from the placental basal plate and survive intracellularly³⁵. Both *L. monocytogenes* and *E. coli* are known to utilize specific receptors to invade intracellularly suggesting that unique bacterial ligand-host receptor interactions might govern bacterial localization^{36, 37}. Genetic factors governing host tendency to harbor latent infections, host responses to microbes, and pregnancy related immune system changes all may further mediate the effect of these bacteria during pregnancy.

In the stratified analysis by gestational age, the data are suggestive that intracellular bacteria occur more commonly in the earliest gestational ages particularly in association with spontaneous preterm birth and raise the question of whether such bacteria can play a role in spontaneous preterm labor and delivery under certain circumstances (for example, emergence from occult niches to contribute to infection). The specific types of organisms present may be different at the extremes of gestational age as prior studies have documented that earlier PTB is more likely to be associated with infection^{20, 38}. However, given the presence of bacteria even in term placentas as well as the absence of bacteria in a large proportion of preterm placentas, one cannot exclude the possibility that such microbes in the basal plate are commensal and exert no pathologic effect, whereas others microbes may activate inflammatory cascades. Further investigations to elucidate the identity, origin, and precise functions of these microorganisms are needed.

Strengths and limitations of the study

Our study has several strengths. First, we employed histological stains optimized for detection of bacteria at other mucosal interfaces, for e.g, the urinary tract, which permit rapid and reliable scoring of bacteria in tissue sections at magnifications of 20×–100×. Morphological evidence of both Gram positive and negative species and of various sizes and shapes underscores that the basal plate is an important area for bacterial colonization at the maternal-fetal interface. Second, we focused on maternal basal plate, which has hitherto not been examined for presence of microbes relative to fetal membranes. Third, the researchers examining the basal plate tissue were not aware of the medical histories nor the gestational ages of the patients' placentas they examined, thus minimizing any bias. Finally, multiple histological stains were used to confirm the presence or absence of bacteria in the basal plates.

A limitation of our study is that we did not employ culture techniques to determine whether bacteria in the basal plate were viable and could be identified with this methodology. Previous studies have indeed detected bacteria in subamniotic and chorion parenchyma, using culture-dependent techniques and culture-independent detection using PCR^{11, 21, 39}. In addition, observational studies cannot address the problem of causality. Our data show that both term and preterm placentas can have intracellular bacteria present without overt infection while a substantial proportion of basal plate specimens even from preterm placentas do not demonstrate bacteria. The lack of significance between term and preterm placentas on the presence intracellular bacteria does not discount the potential pathologic effect of basal plate colonization as a contributor to PTB but raises multiple questions about how host responses to colonization by bacteria. Molecular techniques for detection of bacterial DNA in tissues have shown that bacterial culture methods underestimate the prevalence of bacteria in placentas and amniotic fluids and are themselves limited by their ability to amplify DNA⁴⁰. Recently, it has been shown that species-specific RNA transcriptome signatures allow rapid identification of pathogens⁴¹. It has also been suggested that deep sequencing analysis would allow for the comprehensive characterization of the microbiome of a given body site such as the basal plate as well as detection of low-prevalence species⁴². Such sophisticated analyses are warranted in the future to definitively identify the basal plate bacterial species. Further investigations will collectively determine

the outcome from the presence of intracellular bacteria as well as host-responses to the bacteria within cells in the basal plate.

In conclusion, we report the novel finding of intracellular bacteria in the basal plate in 27% of placental specimens. Our findings suggest that routine placental pathological examination would be optimized by expanding analyses to include a search for bacteria in this important area of maternal fetal interface.

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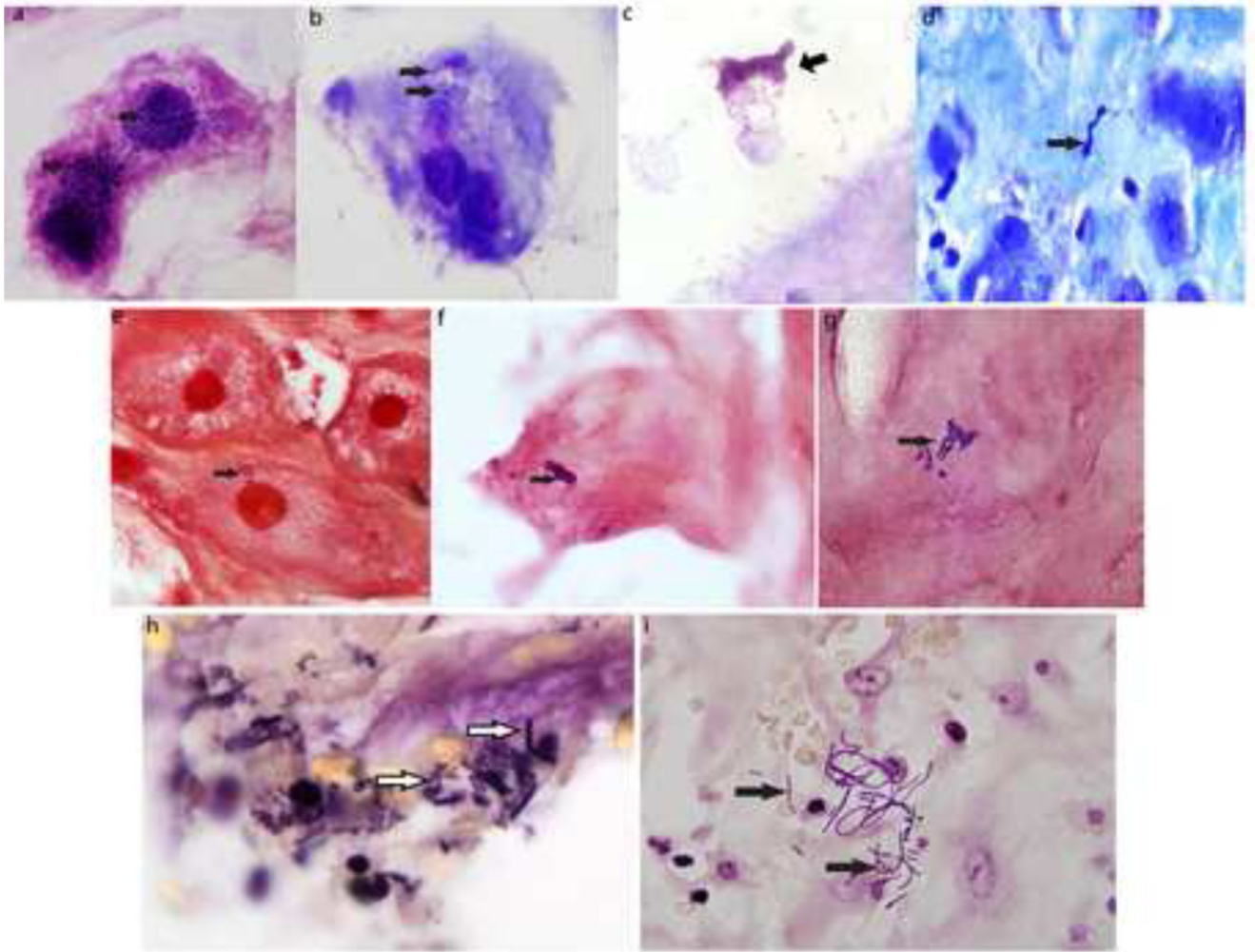


Figure 1. Intracellular bacteria in basal plate
 (a–d) Hema 3 Geimsa stain (e–g) Gram stain (h–t) Brown and Hopps stain: all showing presence of single, clusters, chains, or filaments of intracellular bacteria (arrows).

Table 1

Baseline Maternal Demographic Information

Characteristic	Intracellular Bacteria Present in Basal Plate n=53	Intracellular Bacteria Absent in Basal Plate n=142	p-value
Advanced Maternal Age (SD)	29.8(5.9)	28.4(5.8)	0.12
Black Race (%)	47.2	47.2	0.99
Tobacco Use (%)	9.4	23.9	0.03
Alcohol Use (%)	5.8	11.4	0.29
STI in current pregnancy (%)	20.8	21.8	0.87
History of STI prior to pregnancy (%)	34.6	26.8	0.29
UTI in current pregnancy (%)	18.9	18.3	0.93
Group B Streptococcus infection	21.6	22.9	0.85

STI=sexually transmitted infection; UTI=urinary tract infection

Table 2

Comparison of Presence of Intracellular Bacteria in Preterm Birth and Chorioamnionitis

	Intracellular Bacteria Present in Basal Plate (%)	Intracellular Bacteria Absent in Basal Plate	p-value
Preterm Birth < 37 weeks (n=68)	35.9	34.5	0.86
Chorioamnionitis (n=33)	13.2	18.3	0.40

Table 3

Presence of Intracellular Bacteria in Placentas Stratified by Gestational Age

Gestational Age (weeks)	Intracellular Bacteria Present in Basal Plate (%)	Relative Risk (95%CI)	p-value
37	26.7	Reference	
34–36 (n=26)	15.4	0.6 (0.2–1.5)	0.3
28–33 (n=27)	33.3	1.3 (0.7–2.3)	0.5
<28 (n=15)	40.0	1.5 (0.8–3.0)	0.3

Table 4

Presence of Intracellular Bacteria in Spontaneous Preterm Labor Compared to Term Labor

Gestational Age (weeks)	Intracellular Bacteria Present in Basal Plate (%)	Relative Risk (95%CI)	p-value
37	26.7	Reference	
34–36 (n=17)	11.8	0.4 (0.1–1.7)	0.2
28–33 (n=22)	31.8	1.2 (0.6–2.3)	0.6
<28 (n=11)	54.5	2.0 (1.1–3.8)	0.02