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ABSTRACT

Increasing evidence suggests an association between periodontal disease and adverse pregnancy outcomes. Although infection is considered as a risk factor for preterm delivery, the localization of oral bacteria or their antigens in chorioamnionitis placental tissue has never been demonstrated. This study was devised to test the hypothesis that periodontal pathogens may be present and affect human placenta in cases of chorioamnionitis. Using immunocytochemistry, we have identified the presence of *Porphyromonas gingivalis* antigens in placental tissues. The antigens were detected in the placental syncytiotrophoblasts, chorionic trophoblasts, decidual cells, and amniotic epithelial cells, as well as the vascular cells. There was a substantial increase in immunostaining intensity of the tissues sectioned from women with chorioamnionitis compared to those experiencing normal-term pregnancy, $p < 0.019$ (Mann-Whitney test). These results suggest that *P. gingivalis* may commonly colonize placental tissue, and that the presence of the organism may contribute to preterm delivery.

KEY WORDS: *P. gingivalis*, placenta, preterm, chorioamnionitis, periodontitis.

Localization of *P. gingivalis* in Preterm Delivery Placenta

INTRODUCTION

Periodontal disease and its associated bacteria are thought to be involved in a significant number of systemic conditions, such as cardiovascular disease, diabetes mellitus, and osteoporosis (Garcia *et al.*, 2001; Katz *et al.*, 2001). Although some recent studies failed to demonstrate an association (Michalowicz *et al.*, 2006; Vettore *et al.*, 2008), other reports—including animal, and human epidemiological and interventional studies—have suggested that poor periodontal status may result in preterm delivery and low birthweight (Offenbacher *et al.*, 2006; Goldenberg *et al.*, 2008). The association between preterm labor and bacterial infection is well-established, and it has been reported that as many as 40-50% of cases involve an infection (Lamont, 2003; Klein and Gibbs, 2005). Furthermore, up to 80% of preterm deliveries at fewer than 30 wks of gestation have possible infection (Goldenberg *et al.*, 2000), and there is evidence that these infections precede the development of the pregnancy complications (Gonçalves *et al.*, 2002). Infection, therefore, represents an important and frequent contributor to complications of pregnancy. The route of infection can be either ascending from the urogenital tract, such as occurs with *E. coli* (Goldenberg *et al.*, 2000), or transplacental, such as occurs with *Listeria monocytogenes* and *Salmonella* (Lecuit *et al.*, 2004; Schloesser *et al.*, 2004; Bakardjiev *et al.*, 2005). Bacteria can invade the feto-placental tissue, causing tissue damage and premature labor. Alternatively, adverse outcomes can result from a complex interplay between the bacteria and the host cells and tissues that disrupts normal immune and inflammatory status.

P. gingivalis, a consensus pathogen in periodontal disease, has been detected in amniotic fluid from women identified as having threatened premature labor (preterm premature rupture of membranes without clinical infection or labor and preterm labor with intact membranes) (Leon *et al.*, 2007), although a definitive role for *P. gingivalis* in preterm delivery has yet to be established. However, recurrent bacteremias with *P. gingivalis* and other oral bacteria are common after dental procedures and can occur in persons with periodontitis during simple mastication (Loesche, 1997; Geerts *et al.*, 2002), which would allow *P. gingivalis* to reach the placental tissues by hematogenous spread.

To our knowledge, there are no human studies that have directly identified the presence of periodontal bacteria in placental tissue affected by chorioamnionitis. This missing link is essential in establishing the direct relationship between preterm births and periodontal disease. The purpose of the present study, therefore, is to utilize specific antibodies to demonstrate the presence of *P. gingivalis* in placental tissues.

MATERIALS & METHODS

The study was approved by the University of Florida Health Center IRB.

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Figure 1. Immunohistochemical localization of *P. gingivalis* in human placenta (A, and higher magnification in B) and fetal membrane (C) from women with chorioamnionitis. Bacteria are labeled with polyclonal antibodies to *P. gingivalis* and appear red-brown after immuno-enzymatic color development. Note the presence of immunoreactive *P. gingivalis* in placental syncytiotrophoblasts (ST) and placental arterial endothelial cells (EC), as well as in amniotic epithelial cells (AM), chorion (CH), and decidua (DC). The lowest arrow in (C) shows the presence of a focal inflammatory response with a large inflammatory cell infiltrate, some of which also immunostained for *P. gingivalis*. (D) Pre-immune rabbit serum as a control did not show any red-brown-staining bacteria. Magnifications: A, C, and D, 60X; B, 150X.

Table. Immunohistochemical Staining Grading for *P. gingivalis* (overall score = intensity × extensiveness; adapted after Patel et al., 2008)

Intensity		Extensiveness	
Score	Interpretation ^a	Score (assigned)	Interpretation ^a
0	No staining	0% (0) ^b	No visible areas
1	Faint staining	<10% (10)	Very few stained
2	Moderate staining	10-25% (17.5)	Few staining
3	Strong staining	25-50% (37.5)	Less than half
		50-75% (62.5)	More than half
		75-90% (82.5)	Majority staining
		>90% (90)	All cells staining

^a The intensity and extensiveness were determined per high-power field.

^b Values in brackets are the assigned average of the extensiveness percentage that was used for the overall score calculation.

Tissue

Formalin-fixed paraffin-embedded sections were obtained from placental and fetal membranes from women who had normal-term pregnancies (n = 5) or preterm labor complicated by chorioamnionitis (n = 9) with fewer than 37 wks of gestation. The sections were obtained from the Department of Obstetrics and Gynecology, University of Florida College of Medicine, and Shands Medical Center, Gainesville, Florida.

Chorioamnionitis was diagnosed on the basis of fever (greater than 38°C) and maternal and fetal tachycardia in the absence of other localizing signs of infection.

Antibody Staining

P. gingivalis polyclonal antibodies were raised against formalinized cells of the type strain ATCC 33277 (Yilmaz et al., 2002). This antibody does not react with human cells or with other bacteria at dilutions of 1:500 or greater. Control antibodies were normal rabbit serum and serum raised to *S. gordonii* strain G9B (Lamont et al., 1988). Tissue sections were immunostained with antibodies at a 1:5000 dilution, followed by peroxidase-conjugated secondary antibody and enzyme substrate. Sections were counterstained with hematoxylin and visualized by light microscopy

(Olympus BH2, Center Valley, PA, USA) according to the procedure for paraffin-embedded sections (Koenig and Chegini, 2000).

Data Analysis

Staining intensity was rated according to the following scale: no visible staining = 0, faint staining = 1+, moderate staining = 2+, and strong staining = 3+. Extensiveness was graded semi-quantitatively as 0%, <10%, 10-25%, 25-50%, 50-75%, 75-90%, and >90% of positively stained cells, per high-power field. To compare all of the available data, we assigned an overall score to each case by multiplying the intensity score by the mean percentage of cells stained (extensiveness). This method has been used and verified by others (Luo et al., 2002; Patel et al., 2008). The overall score was used as the basis for statistical

comparison (Table). The Mann-Whitney test was used for the statistical analysis.

RESULTS

Immunostaining by *P. gingivalis* antiserum specifically was associated with placental syncytio-trophoblasts, chorionic trophoblasts, decidual cells, and amniotic epithelial cells, as well as with vascular cells (Fig. 1). There was a substantial increase in immunostaining intensity of the tissues sectioned from women with chorioamnionitis as compared with those experiencing normal-term pregnancy (Fig. 2).

After the staining scores of both groups were compared, the chorioamnionitis group expressed *P. gingivalis* antigens almost 30% more intensely than the normal placenta groups. This difference was statistically significant (Fig. 3, $p < 0.019$).

All normal placentas ($N = 5$) expressed some degree of reactivity with *P. gingivalis* antibodies. The most commonly stained areas in both normal and infected placentas were trophoblasts (60% of all positively stained cells), decidual cells (20%), and vasculature cells (20%). No staining was observed with *S. gordonii* or normal rabbit antibodies.

DISCUSSION

In the present study, we have demonstrated the presence of *P. gingivalis* antigens on trophoblasts, decidual cells, amniotic epithelial cells, and vascular cells, suggesting a possible route of transmission from the vasculature to placental tissue.

Although the staining was present in both normal placentas and placentas affected by chorioamnionitis, the extent to which the latter were positive was 30% higher compared with the normal. The difference in staining between the two groups was statistically significant. As far as we know, this is the first report that documents increased expression of *P. gingivalis* antigens in human placental cells affected by chorioamnionitis.

P. gingivalis is a Gram-negative anaerobe that can invade and survive within epithelial cells (Lamont and Jenkinson, 1998). Infection by *P. gingivalis* disrupts cytokine expression and increases cell proliferation while suppressing apoptosis (Mao *et al.*, 2007). All of these properties could potentially disrupt homeostasis in the placental tissues. It is also potentially important that, during the first 10-12 wks of gestation, the placenta is in a state of physiological hypoxia (James *et al.*, 2006), which would facilitate growth of anaerobes such as *P. gingivalis*.

Figure 2. Immunohistochemical localization of *P. gingivalis* from women with chorioamnionitis (A,B). Normal placenta (C) and control section (D). Bacteria are labeled with polyclonal antibodies to *P. gingivalis* and appear red-brown after immuno-enzymatic color development. (D) Pre-immune rabbit serum as a control did not show any red-brown-staining bacteria. Magnifications: 150X.

Figure 3. Comparison between *P. gingivalis* expression in placental tissues affected by chorioamnionitis ($n = 9$), and normal placenta ($n = 5$). The overall score = intensity $\times$ extensiveness. Adapted after Patel *et al.*, 2008. The error bars represent SD.

Preterm delivery of low-birthweight infants is a major public health concern that contributes to infant mortality and short- and long-term morbidity. Preterm delivery occurs in about 12% of births, although rates are higher (more than 20%) among poor and minority pregnant women. Despite dramatic advances in reproductive and neonatal medicine in general, the rate of

preterm delivery is not decreasing in either developed or developing countries. While several risk factors for preterm delivery have been identified, their predictive power is limited, and there are no effective treatments to prolong pregnancy once preterm labor commences.

Our present findings show that *P. gingivalis* antigens are more frequent and more intense in the chorioamnionitis placenta compared with the normal placenta. This is in accordance with some epidemiological studies that have associated severe periodontal disease with subsequently increased bacterial load and preterm delivery.

We chose to focus on *P. gingivalis*, since this organism is the predominant pathogen in severe cases of periodontal disease, is epidemiologically associated with preterm delivery, and expresses appropriate virulence properties in animal models. However, it is possible, and indeed likely, that other species—such as *F. nucleatum*, *A. actinomycetemcomitans*, *P. intermedia*, *T. forsythia*, and *T. denticola*—are present (Barak et al., 2007). Other relevant factors are immune system competency, as well as the physical integrity of placental tissues and their ability to resist bacterial intrusion. It is important to note that the reactivity to *P. gingivalis* antibodies does not confirm the presence of whole bacteria, but rather their antigens.

In conclusion, accumulating evidence implicates a role for periodontal pathogens such as *P. gingivalis* in pregnancy complications, including preterm delivery. To date, this evidence is based on epidemiological studies and the pathogenic attributes of the organism in animal placentas following infection and in human amniotic fluid (Lin et al., 2003; Leon et al., 2007). Analysis of our data confirms the results of another study (Barak et al., 2007) showing that *P. gingivalis* antigens can be present in the normal asymptomatic human placenta. The significance of this finding remains to be determined, although asymptomatic tissue colonization of bacteria is well-documented. The hypothesis is that *P. gingivalis* bacteremia spreads to the placenta, and infection becomes common. Disease would then occur when there was a change in the overall host-microbe balance, much as in periodontal disease.

Disease outcome following *P. gingivalis* colonization of the placenta may therefore be dependent on many factors, including bacterial genotype and load, host physiology, genetics, and environmental factors.

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