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Microbial invasion of the amniotic cavity in pregnancies with small-for-gestational-age fetuses

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Abstract

Objective: Microbial invasion of the amniotic cavity (MIAC) has been detected in women with preterm labor, preterm prelabor rupture of membranes (PROM), and in patients at term with PROM or in spontaneous labor. Intrauterine infection is recognized as a potential cause of fetal growth restriction; yet, the frequency of MIAC in pregnancies with small-for-gestational-age (SGA) fetuses is unknown. The aim of this study was to determine the frequency, diversity and relative abundance of microbes in amniotic fluid (AF) of women with an SGA neonate using a combination of culture and molecular methods.

Method: AF from 52 subjects with an SGA neonate was analyzed with both cultivation and molecular methods in a retrospective cohort study. Broad-range and group-specific PCR assays targeted small subunit rDNA, or other gene sequences, from bacteria, fungi and archaea. Results of microbiologic studies were correlated with indices of the host inflammatory response.

Results: 1) All AF samples (n = 52) were negative for microorganisms based on cultivation techniques, whereas 6% (3/52) were positive based on PCR; and 2) intra-amniotic inflammation was detected in one of the three patients with a positive PCR result, as compared with three patients (6.1%) of the 49 with both a negative culture and a negative PCR (P = 0.2).

Conclusion: MIAC is detected by PCR in some patients with an SGA fetus who were not in labor at the time of AF collection.

Keywords: 16S rRNA; chorioamnionitis; cytokines; FIRS; IL-6; intra-amniotic infection; intra-amniotic inflammation; molecular microbiology; PCR; pregnancy; SGA.

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Introduction

A small-for-gestational-age (SGA) neonate is usually defined as one whose birth weight is below the 10th percentile for gestational age (GA) [1, 24, 71]. An SGA newborn may be constitutionally small or the consequence of several mechanisms of disease, such as uteroplacental insufficiency, chromosomal abnormalities, congenital infection, genetic syndromes, etc. [76]. Therefore, SGA is considered one of the ''great obstetrical syndromes'' because it has multiple etiologies, a long preclinical phase and the other criteria that define these syndromes [17, 55, 56].

Proposed mechanisms of disease of SGA include endothelial cell dysfunction [5], an anti-angiogenic state [9, 10, 18, 25, 62, 74], inadequate physiologic transformation of the spiral arteries [7, 22] and a maternal intravascular exaggerated inflammatory response [29, 34, 46, 70, 72]. Perinatal infections, mainly of viral or parasitic origin (i.e., cytomegalovirus, rubella, herpes, toxoplasmosis, etc.) [26, 28, 33, 37, 48, 51, 52, 73], have also been implicated as a cause of SGA.

Experimental studies have demonstrated that chronic infection/inflammation during pregnancy may result in an SGA fetus in hamsters [11, 12] and mice [38, 78]. In humans, maternal microbial infections during pregnancy have been associated with impaired fetal growth [3, 13, 19, 21, 42, 44,

45]. However, it is unknown if microbial invasion of the amniotic cavity (MIAC) with bacteria or fungi could be associated with SGA neonates in humans. A literature search in PubMed performed in March 2010 using different combinations of the keywords: ''small-for-gestational age'', ''SGA'', ''intra-uterine growth retardation'', ''IUGR'', ''infection'', and ''amniotic fluid'' limited to humans and published in English did not reveal any study addressing this question.

The objectives of this study were to determine the frequency, taxonomic diversity and relative abundance of microbes in amniotic fluid (AF) of women with an SGA neonate using a combination of cultivation and molecular methods.

Methods

Study population

A retrospective cohort study was conducted of patients with an SGA neonate (defined below) who met the following inclusion criteria: 1) singleton gestation; 2) GA between 24 and 42 weeks; and 3) amniocentesis with microbiological studies of AF. Exclusion criteria were: 1) active term or preterm labor; 2) ruptured membranes; 3) preeclampsia; or 4) a major fetal chromosomal and/or congenital anomaly. Patients in labor and/or with rupture of membranes were excluded because these conditions have been associated with a high rate of MIAC and could confound the research question of this study.

All women provided written informed consent prior to the collection of biological samples. The utilization of samples and clinical data for research purposes was approved by the Institutional Review Boards of Sotero del Rio Hospital, Azienda Ospedaliera of Padova, Wayne State University, the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD/NIH/DHHS), and Stanford University.

Definitions

An SGA neonate was defined by sonographic estimated fetal weight below the 10th percentile for GA [1, 24] and confirmed by neonatal birthweight. Histologic chorioamnionitis was diagnosed based on the presence of inflammatory cells in the chorionic plate and/or chorioamniotic membranes [32, 53]. Acute funisitis was diagnosed by the presence of neutrophils in the wall of the umbilical vessels and/or Wharton's jelly using criteria previously described [49]. Intra-amniotic inflammation was defined by an AF interleukin (IL)-6 concentration >2.6 ng/mL [81].

Sampling procedures

Patients with an SGA fetus were offered amniocentesis for genetic indications, to assess the microbial status of the amniotic cavity and to assess fetal lung maturity. In patients undergoing cesarean delivery, AF was retrieved intra-operatively. AF was transported in a capped sterile syringe to the clinical laboratory where it was cultured for aerobic and anaerobic bacteria, including genital mycoplasmas, as described previously [15]. A white blood cell (WBC) count [64] and Gram stain [58] of AF were also performed shortly after collection using methods previously described. Shortly after the amniocentesis, AF not required for clinical assessment was centrifuged at $1300\times g$ for 10 min at 4°C, and the supernatant was aliquoted into gamma-irradiated non-pyrogenic DNase/RNase-free cryovials (Corning, Acton, MA, USA), and immediately frozen at −70°C. AF IL-6 and matrix metalloproteinase (MMP)-8 concentrations were determined using a specific and sensitive immunoassay which had been validated for AF [43]. IL-6 and MMP-8 determinations were performed after all patients were delivered and were not used in clinical management.

Genomic DNA extraction

AF that was not required for clinical purposes ($\approx$200 μL of each AF sample) was shipped on dry ice to Stanford, CA, USA, where genomic DNA was extracted as described previously [16]. Extracted DNA was eluted into a final volume of 100 μL of QIAamp® AE buffer and stored at −20°C or colder until thawing for molecular analyses. Strategies to prevent, detect and neutralize potential contamination were implemented at critical steps [4], according to a previously described protocol. This included mock extraction blanks (sterile water processed in parallel, and in the same manner as AF samples) to monitor potential contamination (at least one mock was included per 17 processed samples) [15].

Qualitative analysis by end-point PCR

DNA from each AF sample was analyzed by end-point PCR using broad-range bacterial 16S ribosomal DNA (rDNA) primers, and by group-specific end-point PCR using primers specific for six taxonomic groups, including *Candida* sp. (Table 1 [6, 14, 36, 50, 77, 82]) PCR reactions, screening of PCR products by gel electrophoresis, and purification and cloning of amplicons from broad-range PCR were performed as described [16]. Sequencing of amplicons directly from group-specific PCRs, and of recombinant clones from broad-range PCR (up to 10 clones per reaction) was performed as described [15].

Sequence alignment and phylogenetic analysis

Forward and reverse sequence reads were assembled into contigs as described [15]. Assembled sequences from group-specific PCR were queried against NCBI's GenBank database using a basic local alignment search tool (BLAST) algorithm [2] to confirm specificity. Assembled sequences from broad-range end-point PCR were aligned and subjected to phylogenetic analysis as described [15]. After removal of vector, human, and poor-quality sequences from the alignment, a neighbor-joining tree was generated based on Felsenstein correction and 682 unambiguous filter positions. Phylotypes were defined using a 99% sequence similarity threshold, which approximates a species-level classification.

Quantitative analysis by real-time PCR

DNA from each sample was analyzed by means of two real-time PCR assays, each of which was designed to amplify in a specific manner and quantify 16S rDNA of domain *Bacteria* or domain *Archaea* (Table 1). Reactions were carried out as described [16].

Statistical analysis

Comparison between continuous variables was performed with the Mann-Whitney *U*-test. Comparison of proportions was performed using Fisher's exact tests. A P-value <0.05 was considered statistically significant. Analysis was performed with SPSS, version 12 (SPSS Inc., Chicago, IL, USA).

Table 1 PCR assays used in this study.

Approximate taxonomic level	End-point PCR taxonomic specificity	Lower detection limit (gene copies/μL)	Oligonucleotide name	Use	Sequence (5′→3′)	Gene target	Reference
Domain	*Bacteria*	100	Bact-8FM	FP	AGAGTTTGATCMTGGCTCAG	16S rDNA	[50]
			Bact-806R	RP	GGACTACCAGGGTATCTAAT		[77]
Genus	*Ureaplasma*	10	Urease185F	FP	GCTGCTGACGTTGCAAGAAG	Urease gene	[16]
			Urease756R	RP	CTCCTGGTTCAAAACGAATAGC		[16]
Genus	*Fusobacterium*	100	Fuso-422F	FP	CGGAATGTAAAGTGCTTTC	16S rDNA	[16]
			Fuso-710R	RP	CCCATCGGCATTCCTAC		[16]
Genus	*Sneathia/Leptotrichia*	10	SsLa-140F	FP	TAGACTGGGATAACAGAGG	16S rDNA	[16]
			SsLa-406R	RP	AGTCCTAAAACCTTCTTCACAC		[16]
Species	*Streptococcus agalactiae*	10	Sag059F	FP	TTTCACCAGCTGTATTAGAAGTA	*cfb*	[30]
			Sag190R	RP	GTTCCCTGAACATTATCTTTGAT		[30]
Species	*Mycoplasma hominis*	10	Mh-148F	FP	CAATGGCTAATGCCGGATACG	16S rDNA	Mod. from [82]
			Mh-463R	RP	GGTACCGTCAGTCTGCAATC		Mod. from [82]
Genus	*Candida*	10	Cand-ITS2-42F	FP	GGGTTTGCTTGAAAGACGGTA	ITS2	[16]
			Cand-ITS2-125R	RP	TTGAAGATATACGTGGTRGACGTTA		[16]
	Real-time PCR taxonomic specificity	Dynamic range (gene copies/μL)					
Domain	*Bacteria*	15–1e8	Bact-8FM	FP	AGAGTTTGATCMTGGCTCAG	16S rDNA	[50]
			Bact-338K*	Probe	CCAKACTCCTACGGGAGGCAGCAG		[50]
			Bact-515R	RP	TTACCGCGGCKGCTGGCAC		[6]
Domain	*Archaea*	100–1e8	Arch333F	FP	TCCAGGCCCTACGGG	16S rDNA	[36]
			Univ-515F*	Probe	GTGCCAGCMGCCGCGGTAA		[6]
			Arch958R	RP	YCCGGCGTTGAMTCCAATT		[14]

FP = forward primer, RP = reverse primer, Probe = TaqMan probe.
*Conjugated on the 5′ end to 6-carboxyfluorescein, and on the 3′ end to 6-carboxy-tetramethylrhodamine.

Table 2 Demographic and clinical characteristics of the study population.

Variables	Patients with SGA (n = 52)
Maternal age (years)	30 (23–34)
Ethnicity	
African American	26 (50)
Caucasian	20 (28.5)
Hispanic	6 (11.5)
Pre-pregnancy BMI (kg/m^2)	22.3 (20.3–27.1)
Gestational age at amniocentesis (weeks)	36.9 (34.5–39)
Gestational age at delivery (weeks)	37.5 (34.6–39)
Birthweight (g)	2245 (1690–2587)

Data presented as median (interquartile range) or number (%).
BMI = body mass index.

Results

Demographic and clinical characteristics of the 52 patients enrolled in the study are presented in Table 2.

Microbial invasion of the amniotic cavity in SGA

All samples were negative for MIAC based on cultivation methods whereas 5.8% (3/52) of samples were positive for MIAC based on PCR methods. Two of the three PCR-positive samples were detected by broad-range PCR: one sample had evidence of *Streptococcus agalactiae* (10 clones; 100% identity to type strain ATCC 13813^T), and one had evidence of *Staphylococcus epidermidis* (2 clones; 100% identity to type strain ATCC 14990^T). The other sample with molecular evidence of MIAC was positive by group-specific PCR for *Candida* sp. In addition, group-specific PCR for *Streptococcus agalactiae* was also positive in the sample that yielded this species by broad-range PCR. This was also the only sample with a high microbial rDNA abundance (e.g., >500 genes/μL AF) based on broad-range real-time bacterial PCR, which estimated 16S rDNA abundance in this sample to be $\sim 10^5$ genes/μL of AF. Table 3 displays the clinical information of the cases that were positive by PCR.

Assessment of the intra-amniotic inflammatory response

Intra-amniotic inflammation was detected in one of the three patients with a positive PCR (Table 3). Among the 49 patients with both a negative AF culture and a negative PCR, three cases (6.1%) had intra-amniotic inflammation (P = 0.2). The median concentrations of the different markers of intra-amniotic infection/inflammation (i.e., WBC count and glucose, IL-6 and MMP-8 concentration) were not significantly different between patients with a positive PCR and those with negative cultures and negative PCR (AF WBC: P = 0.4, glucose: P = 0.1, IL-6: P = 0.1, and MMP-8: P = 0.4).

Short-term neonatal outcome

In the case that was PCR-positive for *Candida* sp., the neonate had an elevated C-reactive protein (CRP) in the first

Table 3 Clinical characteristics of the three cases with a positive amniotic fluid PCR.

Patient no.	Microbe	GA at AC (weeks)	AF WBC (cell/mL)	AF glucose (mg/dL)	AF IL-6 (ng/mL)	AF MMP-8 (ng/mm^3)	Placental pathology	GA at delivery (weeks)	Birthweight (g)
1	*Candida* sp.	32.3	7	41	0.64	3.81	No inflammation	32.3	1370
2	*Staphylococcus epidermidis*	35.3	4	44	0.41	1.51	No inflammation	35.3	1730
3	*Streptococcus agalactiae*	39.9	4	50	44.81	32.65	Villous tree alterations	39.9	2670

GA = gestational age, AC = amniocentesis, AF = amniotic fluid, WBC = white blood cell.

day of life; he received antibiotics (ampicillin and gentamicin) for 4 days and the CRP concentration subsequently normalized and blood cultures were negative. In the case with *Staphylococcus epidermidis*, the neonatal WBC count and differential were normal and blood cultures were negative. Of note, the managing physicians were not aware of the results of PCR which was performed later.

In the case that was PCR-positive for *Streptococcus agalactiae*, the neonate had an uneventful outcome and was discharged home with the mother.

Discussion

Principal findings of the study

Using cultivation techniques, none of the patients had microorganisms detected in the AF; however, by including molecular methods in our approach, we found MIAC in ~6% of patients with an SGA neonate.

Detection of microbial invasion of the amniotic cavity

The AF in normal pregnancy is considered sterile in the majority of cases. However, MIAC has been demonstrated in 18% of patients in spontaneous labor at term with intact membranes [63], 34% of women with prelabor rupture of membranes (PROM) at term [61], 13% of women presenting with an episode of preterm labor [23], 32% of women with preterm PROM [23], and 9% of women with a short cervix [27]. Among women with cervical insufficiency, the prevalence of MIAC is about 50% [59]. However, all these estimates are based upon cultivation techniques and rely on the ability to provide adequate conditions required for the growth of microorganisms in the laboratory.

Molecular methods offer a cultivation-independent approach to microbial detection, and various types of molecular assays provide relative advantages. For example, broad-range PCR assays that target rDNA with universal primers enable detection and characterization of diverse microbial taxa, including previously-unknown species [54]. On the other hand, group-specific PCR assays that amplify gene sequences unique to smaller groups of related taxa are often more sensitive; however, the specific microbial groups must be suspected in advance. Both approaches yielded positive findings in the current study.

Our group previously reported that specific PCR assays for *Ureaplasma urealyticum* are more sensitive than cultivation for this species in AF of patients with preterm labor and intact membranes [80], preterm PROM [79], and cervical insufficiency [8]. We have also employed a combination of broad-range and specific PCR assays for bacteria and fungi, and have demonstrated that the combination of culture and molecular methods allows improved detection of MIAC [15, 16]. Importantly, an intrauterine inflammatory response is associated with the presence of microbial DNA in the AF, even in the settings of a negative culture [15, 16, 31, 79, 80]. Such findings provide evidence that a positive PCR-based assay has biological significance [20].

MIAC in patients with SGA

We have not been able to identify any prior study that has systematically examined MIAC in SGA with cultivation or molecular methods. Most studies have focused on the presence of selected viruses, such as Cytomegalovirus, or specific micro-organisms, such as *Toxoplasma gondii* [26, 28, 37, 48, 51, 52, 73].

Our findings suggest that ~6% of women with SGA neonates have MIAC detected by molecular techniques, and that these cases escape detection by cultivation techniques routinely employed in a clinical laboratory supporting an obstetrical service. The organisms identified included *Streptococcus agalactiae* (group B streptococci), *Candida* sp. and *Staphylococcus epidermidis*.

One interesting sample (containing *Streptococcus agalactiae*) was positive both by broad-range PCR and by group-specific PCR, and was found to have a high microbial burden based on 16S rDNA copy number, as measured by real-time PCR. This case had evidence of a robust immune response, and was associated with the highest concentrations of both IL-6 (44.8 ng/mL), and MMP-8 (32.6 ng/mL) in our study population (the next highest measurements of each marker were 8.78 ng/mL for IL-6, and 1.03 ng/mL for MMP-8). These two parameters have been associated with the presence of intra-amniotic infection in previous studies [39–41, 57, 66, 81]. In addition, our prior studies found microbial rDNA levels to be inversely correlated with GA at delivery [15, 16].

Two other samples were positive by PCR for a single taxon each: one for *Candida* sp., and one for *Staphylococcus epidermidis*. In these cases, the concentrations of IL-6 and MMP-8 were not elevated; therefore, it is possible that PCR detected MIAC at an early stage prior to the development of a significant host response, or that one or more of these taxa represent contamination, despite the rigorous method of AF collection. These samples were collected in the operating room at the time of cesarean delivery, and the patients were not in labor.

Implications of the findings

Our results suggest that a small group of SGA fetuses have subclinical MIAC, and that in some instances, this is associated with an intra-amniotic inflammatory response as determined by the AF concentrations of IL-6 and MMP-8. It is also interesting that the patient with a high microbial burden and a robust response was not in labor; however, a cesarean section was performed at term. It seems that not all cases of MIAC are associated with the spontaneous onset of labor, even though the natural history of the patient was interrupted by a cesarean section. Whether micro-organisms may exist in the amniotic cavity for a period of weeks without eliciting an inflammatory response remains to be determined. Similarly, whether micro-organisms can multiply in AF, eliciting an inflammatory response, but not lead to the initiation of labor or rupture of membranes is also possible.

Strengths and limitations of the study

This is the first study to examine, in a systematic manner, AF from pregnancies with SGA neonates to determine the presence or absence of microbial invasion using both cultivation and molecular methods. We also examined indices of the intra-amniotic inflammatory response (IL-6 and MMP-8).

One limitation of our study was its sample size (n = 52). However, prior to this study, there was no estimate of the rate of MIAC in this clinical phenotype. The conventional view has been that MIAC is associated with spontaneous preterm labor (with intact or ruptured membranes) [60, 61, 65, 67–69, 80], cervical insufficiency [35, 47, 59], or short cervix [27, 75], but not with indicated causes of preterm delivery, such as SGA and preeclampsia.

Conclusions

MIAC was detected using molecular techniques, but not cultivation techniques, in association with ~6% of SGA neonates. The detection in one case of *Streptococcus agalactiae* (group B streptococci) was associated with a demonstrable AF inflammatory response. The role of microbes in the pathophysiology of SGA requires further study. Studies to determine the frequency, diversity, and relative abundance of micro-organisms in AF from normal pregnant women in the mid-trimester of pregnancy and from women at term not in labor are in progress. We believe that such studies will assist in placing the information presented in this study in context.

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