Amniotic Fluid Defensins: Potential Markers of Subclinical Intrauterine Infection

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Human neutrophil peptides 1–3 (defensins) are granule constituents released from activated neutrophils. We hypothesized that amniotic fluid (AF) defensin levels are elevated in preterm labor (PTL) patients with subclinical intrauterine infection (IUI). AF samples were obtained from 203 pregnant patients with varying clinical characteristics. Defensin levels were measured by enzyme-linked immunosorbent assay. Median AF defensin levels were fourfold to 24-fold higher in patients with IUI than in preterm and term controls. Among patients with subclinical IUI, the degree of AF defensin elevation was greater in those with a positive AF culture. AF defensin levels increased exponentially with increasing severity of histologic chorioamnionitis. An AF defensin level of >2,500 ng/mL identified 88% of patients with a positive AF culture, whereas a level of >400 ng/mL identified 85% of all infected patients. AF defensin levels accurately identify patients with subclinical IUI, as defined by a positive AF culture or placental histology.

Preterm birth remains the major cause of neonatal morbidity and mortality. Although the etiology of preterm labor (PTL) and delivery is unknown, there is overwhelming epidemiological, pathological, and immunologic evidence to support infection of the uteroplacental unit as a causative factor in a substantial proportion of cases of preterm birth [1, 2]. It is hypothesized that organisms from the lower reproductive tract ascend and invade the decidua, leading to an inflammatory response. The resultant prostaglandin production in turn initiates PTL and eventual delivery.

The diagnosis of intrauterine infection (IUI) in the absence of systemic maternal symptoms is difficult and currently relies on culture of the amniotic fluid (AF) obtained by amniocentesis. Limitations to this method of diagnosis include the delay necessary for bacterial culture as well as the inherent risk involved in performing an invasive procedure. In addition, culture is a less than optimal ''gold standard,'' as zero to 24% of patients have positive culture results, while 19% to 74% will have histologic evidence of placental infection after birth [3–9]. It is likely that positive AF cultures identify only those at the end stage of the infectious process and vastly underestimate the true rate of infection.

Surrogate markers of infection may enhance diagnostic strategies for IUI. Therefore, we have initiated studies measuring various neutrophil products in AF as markers for subclinical IUI. The level of lactoferrin, a constituent of the neutrophil secretory granule, predicted IUI with great accuracy before the gestational age of 32 weeks [10]. However, the observation of increasing levels after 32 weeks in uninfected nonlaboring patients limits the clinical application of this test.

Human neutrophil peptides 1–3 (defensins) are antimicrobial peptides found exclusively in the azurophilic granule of the neutrophil [11]. Defensins are the most abundant protein within the neutrophil and are highly stable with regard to pH, proteolysis, and prolonged storage. Therefore, they are ideal markers to utilize in a research setting. Previous studies have shown that defensin levels in plasma are significantly elevated in patients with sepsis and meningitis [12]. We hypothesize that defensins will be excellent markers of subclinical IUI at any gestational age.

Materials and Methods

Patient recruitment. AF samples were obtained from 203 pregnant patients with intact membranes and no clinical evidence of chorioamnionitis. The patients were stratified in the following five groups based on clinical presentation.

Group 1 (term, no labor) included patients at ⩾37 weeks of gestation who were not in labor and were undergoing amniocentesis for fetal lung maturity prior to scheduled elective repeated cesarean deliveries ($n = 50$).

Group 2 (term, labor) included patients at ⩾37 weeks who were in active labor and undergoing cesarean section deliveries for obstetrics indications prior to membrane rupture. AF was obtained during the cesarean delivery via amniocentesis of the intact membranes before delivery of the infant ($n = 17$).

Group 3 (preterm, no labor) included patients at 14–36 weeks' gestation who were not in labor and were undergoing amniocentesis for genetic evaluation or AF bilirubin studies

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($n = 81$). Their fetuses were subsequently found to have a normal karyotype, and the bilirubin study findings were not suggestive of anemia.

Group 4 (PTL with subclinical IUI) included patients in PTL at 24–34 weeks of gestation with intact membranes and subclinical IUI. Subclinical IUI was defined as a positive AF culture or histologic chorioamnionitis without clinical signs of infection (specifically, temperature ≥37.8°C, maternal or fetal tachycardia, or uterine tenderness) ($n = 20$).

Group 5 (PTL without IUI) included patients in PTL at 24–34 weeks' gestation with intact membranes and without subclinical IUI. These patients had negative AF cultures and negative placental histology ($n = 2$) or negative AF cultures and did not deliver within 72 hours of the amniocentesis ($n = 33$).

Exclusion criteria for all groups included abnormal karyotype, maternal isoimmunization, multiple gestation, evidence of fetal membrane rupture by examination, diabetes, treatment with antibiotics within the previous 7 days, and presence of any condition requiring antimicrobial treatment. Exclusion criteria for patients presenting in PTL were a gestational age of <24 weeks or >34 weeks and a cervical dilatation of >4 cm. Gestational age was determined by the best available obstetric criteria. Patients were also excluded if they had clinical evidence of chorioamnionitis, as defined by Gibbs et al. [13]. PTL was defined as the presence of regular uterine contractions with a frequency of 10 contractions per hour and observed cervical change.

Microbiology and histology. AF was cultured for aerobic and anaerobic bacteria, mycoplasmas, *Neisseria gonorrhoeae,* and *Chlamydia trachomatis* as previously described [14]. The remaining fluid was immediately centrifuged at 500–800*g* for 10 minutes, and the supernate was stored at −70°C for later evaluation.

Placentas were collected from all patients who delivered within 72 hours of amniocentesis and were processed as previously described [14]. Histologic chorioamnionitis was defined by the criteria of Salafia et al. [15]. The presence of inflammation of grade 1 (inflammatory cell invasion of at least five polymorphonuclear neutrophils into the amnion, chorion, subchorionic fibrin, or inner third of the umbilical vein wall) or greater in any of the three pathological sections was used to define histologic chorioamnionitis.

Sections were obtained from the fetal membranes, full-thickness placenta, and umbilical cord near the placenta. Each section was scored on a scale of 1 to 4, based on the severity of inflammation. Each placenta was given a total histologic inflammation score of 1–12, determined by addition of the scores for the three different samples. All samples were examined by the same pathologist, who was blinded to the patient's clinical course.

Defensin measurement. AF samples were thawed in batch, and the defensin levels were determined by a double-sandwich ELISA described previously by Panyutich et al. [16]. In brief, Nunc-immunoplate-I microtiter plates were coated with 100 mL of anti-human neutrophil peptide type 1 (anti-HNP-1) monoclonal antibody D1-1 in 0.1 M of Na_2CO_3 buffer (pH, 9.6) at room temperature overnight. Plates were washed in distilled water and blocked with 1% gelatin in 20 mL of Tris-HCl and 500 mL of NACO (pH, 7.5) for 1 hour at room temperature. Samples diluted in Tris-buffered saline (TBS) with .01% CETAB were added and incubated for 2 hours at room temperature.

A second anti-HNP-1 monoclonal antibody (D1–11) in PBS with .01% CETAB, labeled with biotin, was added to the wells for 1 hour at room temperature. The wells were washed four times and incubated for 1 hour with avidin-peroxidase diluted 1/4,000 in TBS with 1% gelatin. The wells were then washed four times. The plates were developed by the addition of 100 μL of O-phenylenediamine at a concentration of 0.2 mg/μL in 20-mM citrate buffer (pH, 4.7), containing 0.25 mM of 30% H_2O_2.

After 5 minutes at room temperature, the reaction was stopped with 50 μL of 2.5-*M* H_2SO_4, and the absorbance was read at 496 on a Bioteck HDi900 miniplate reader (Bioteck, Winooski, VT). Standard curves were generated with use of purified HNP1, and results were expressed as ng of HNP1 per mL of AF. Samples were originally diluted 1:100, with increasing dilutions performed if the original sample measurement exceeded the highest point on the standard curve.

Statistical analysis. Data were analyzed with use of the statistical program SPSS for Windows (Release 6.0; SPSS, Chicago). The Mann-Whitney *U* test was used for nonparametric comparisons of AF defensin levels in the various groups. The Bonferroni correction was used to account for multiple comparisons. The association of gestational age with AF defensin levels and the comparison between pathology score and defensin level were made by means of linear regression and the Spearman correlation. Both methods were utilized since the data from group 3 were normally distributed but those from group 4 were not.

Results

Defensin levels in the various patient groups are presented graphically in figure 1 and descriptively in table 1. To ascertain whether we could compare patients at varying gestational ages, we measured defensin levels in AF samples from 14 to 36 weeks. Our comparisons were valid in that we found no significant increase in AF defensin levels in group 3 patients with increasing gestational age ($R^2 = 5 \times 10^{-7}$).

To assess the impact of labor on AF defensin levels, we compared term patients undergoing repeated cesarean section vs. those undergoing cesarean section who were in active labor. No statistical difference was noted between these two groups; however, both groups had higher defensin levels than patients presenting with idiopathic preterm labor without evidence of

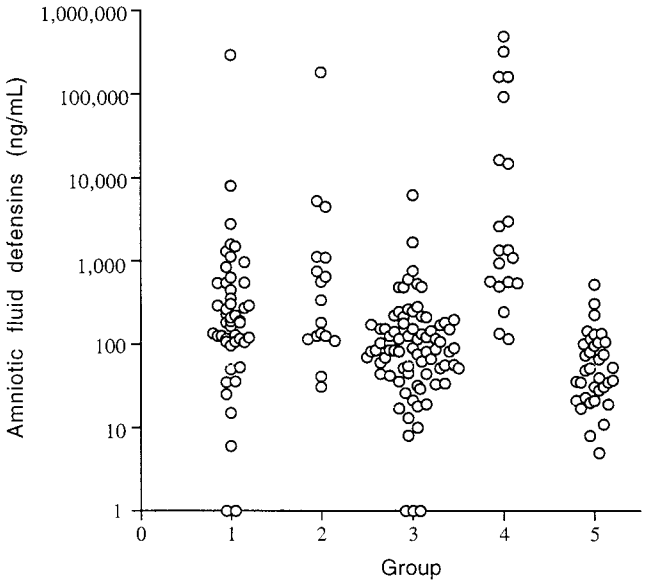

Figure 1. Defensin concentrations in amniotic fluid from women in study group 1 (term, no labor; $n = 50$), group 2 (term, labor; $n = 17$), group 3 (preterm, no labor; $n = 81$), group 4 (preterm labor, with intrauterine infection [IUI]; $n = 20$), and group 5 (preterm labor, without IUI; $n = 33$). Comparisons were made between groups by means of nonparametric techniques, with correction for multiple comparisons.

infection ($P < .001$), suggesting a modest impact of the parturitional process on AF defensin levels.

Median AF defensin levels in patients in PTL with subclinical IUI were fourfold to 24-fold higher than in the other patient groups. All comparisons between these other groups and patients in PTL with subclinical IUI were statistically significant ($P < .001$), except for those undergoing cesarean section during labor at term ($P < .03$). No significant difference was noted between the defensin levels of patients in PTL without IUI and those of the preterm patients not in labor.

Figure 2 demonstrates defensin levels in AF from women with IUI. Median defensin levels in women with a positive AF culture ($n = 8$) were 20-fold higher than in women with histologic chorioamnionitis but a negative AF culture ($n = 12$). Defensin levels were significantly greater in women with a positive AF culture, when compared with all other patient groups ($P < .001$). Figure 3 presents the association between defensin levels and pathology score. A logarithmic increase in defensin levels was observed with increasing severity of placental inflammation (Rho = .80).

Table 2 presents descriptive statistics for the performance of AF defensins in predicting IUI in patients with varying clinical presentations, microbiological findings, and histologic findings. The results for patients with a positive AF culture as well as the entire infected group (as defined by a positive AF culture or positive histology) were based on data for the 55 patients presenting in PTL who were being evaluated for infection. Prediction of pathology score was based on data for the 20 patients with positive histology. Threshold defensin values for the various parameters were based on the best sensitivities and specificities obtained with varying defensin levels. A pathology score of three appeared to be a natural breakpoint for defining which patients could have infection/inflammation as a consequence rather than a cause of labor.

Discussion

In this study we found an excellent correlation between IUI and AF defensin levels. Higher levels of defensins were observed in patients with positive AF cultures. In addition, there was a close correlation between the degree of placental inflammation and AF defensin levels. Labor appeared to have a minimal impact on AF defensins, as evidenced by the slightly increased levels in term laboring patients.

However, our data do not implicate labor alone as the cause of elevated AF defensin levels, as these levels were not signifi-

Table 1. Amniotic fluid defensin concentrations (ng/mL) in five groups of pregnant women with intact membranes.

Patient group	*n*	Median ± SEM	Range	25%–75%[§]
Group 1: term, no labor	50	189 ± 5,991*	0–294,000	107–540
Group 2: term, labor	17	338 ± 10,744[†]	31–183,500	123–1,100
Group 3: preterm, no labor	81	87 ± 81*	0–6,259	47–172
Group 4: preterm, IUI	20	1,352 ± 29,379	116–496,000	550–55,557
Positive AF culture	8	15,808 ± 60,333[‡]	116–496,000	2,817–128,750
Negative AF culture	12	753 ± 28,471	135–327,500	519–1,352
Group 5: preterm, without IUI	30	49 ± 17*	5–522	24–105

NOTE. AF = amniotic fluid; IUI = intrauterine infection.
* $P < .001$ vs. group 4.
[†] $P < .03$ vs. group 4.
[‡] $P < .05$ vs. negative AF culture.
[§] Interquartile range: values between the 25th and 75th percentiles.

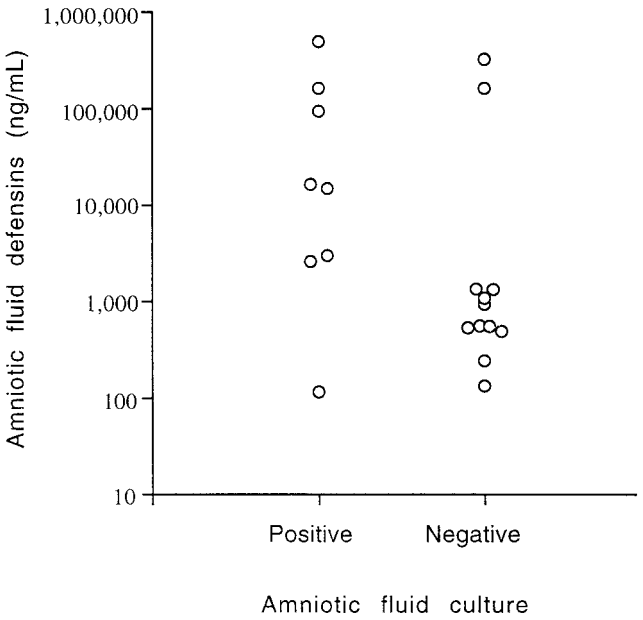

Figure 2. Defensin concentrations in amniotic fluid from patients in preterm labor with an intrauterine infection, separated by amniotic fluid culture positivity or negativity (both groups had significant placental histology). Comparisons were made between groups with use of nonparametric techniques.

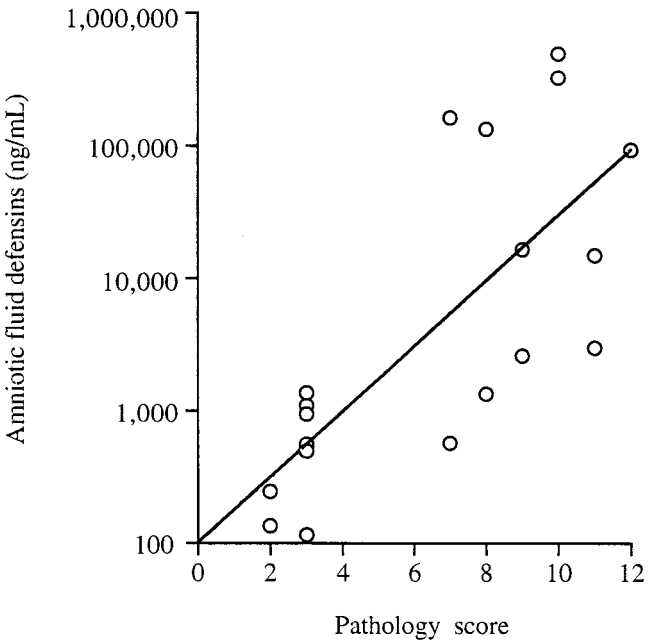

Figure 3. Correlation of pathology score and amniotic fluid defensin concentrations.

cantly different in term laboring vs. term nonlaboring patients (group 1 vs. group 2) and AF defensin levels were lower in term laboring patients than in patients in PTL with culture-proven IUI.

Although our study numbers are small, the AF defensin level appears to be highly accurate in predicting culture-positivity for IUI. The sensitivity of the defensin level in identifying a patient with a positive AF culture was 88% when a threshold value of 2,500 ng/mL was used. This is superior to the 29% to 70% reported for gram staining and is comparable to the 82% to 92% reported for AF glucose levels [17–23].

In our patient group, gram staining detected 75% of infected patients, and the combination of AF defensin levels and gram staining detected all patients with a positive culture. Our specificity of 97% approaches that reported for gram staining and is superior to the 75% to 92% reported for AF glucose level. The two patients with negative cultures and AF defensin levels of >2,500 ng/mL had pathology scores of 10 and 12, respectively, suggesting that both patients had a significant infection. Lowering the threshold value for AF defensin level to 400 ng/mL identified 85% of patients with either positive culture or positive placental histology but had no effect on the specificity, as only one patient presenting in PTL without infection had an AF defensin level of >400 ng/mL.

AF defensins compare favorably to inflammatory cytokines, such as IL-6, as markers of infection. In this same population, Greig et al. reported that IL-6 identified 100% of infected patients, with a specificity of 88% [14]. However, in our patients with IUI, absolute amounts of AF defensins were 500 times greater than the AF IL-6 level (1,351 ng/mL vs. 2.6 ng/mL), and differences between infected and uninfected patients in PTL were 15-fold for IL-6 and 28-fold for defensins.

When compared to levels of other cytokines such as IL-1, IL-8, and tumor necrosis factor, levels of AF defensins are 17–1,500-fold higher in women with a positive AF culture [24–26]. Quantitative differences between defensins and other cytokines as well as the larger discretionary zone between normal and abnormal patients make development of a rapid bedside test such as a dipstick much more feasible with defensins. The abundance of defensins makes it more likely that elevated levels will be present in adjacent or communicating compartments.

Previous work has demonstrated elevated plasma defensin levels in patients with meningitis [12]. If the elevated defensin

Table 2. Descriptive statistics for use of amniotic fluid defensin concentrations to identify varying microbiological or histologic findings in patients presenting in preterm labor.

Patient group, AF defensin level (ng/mL)	Identification of IUI	
	Sensitivity	Specificity
Culture-positive	7/8	45/47
>2,500	88%	96%
All infected	17/20	34/35
>400	85%	97%
Pathology score >3	9/11	9/9
>2,500	82%	100%

levels in AF produce elevated levels in body fluids communicating with the amniotic cavity, i.e., maternal blood or vaginal secretions, diagnosis may not depend on the highly invasive amniocentesis.

It is likely that the elevated AF defensins serve an important physiological function. In vitro, defensins have activity against many gram-positive and gram-negative bacteria, spirochetes, fungi, and viruses [11]. In vivo, this interaction is thought to be limited to the point of neutrophil-organism contact, as activity is abolished when defensins bind serum proteins. This localization of defensin-mediated activity is crucial because defensins also damage eukaryotic cells [27, 28].

The protein-buffering capacity of defensins in blood is ~100,000 ng/mL, making it unlikely that defensins would cause significant cytotoxicity in this compartment [12]. AF protein levels are 10% of serum levels, thereby reducing buffering capacity to only 10,000 ng/mL [29]. In this study, 30% of all patients with IUI had defensin levels >10,000 ng/mL, suggesting that defensin-mediated cytotoxicity could occur. We speculate that this could lead to fetal membrane damage and contribute to the increased risk of fetal membrane rupture that is seen in patients presenting with PTL and positive AF cultures [30].

Furthermore, if AF defensin levels correlate with levels found in fetal blood or CSF, it is plausible that defensins contribute to the increased risk of fetal morbidity, i.e., periventricular leukomalacia or spastic cerebral palsy, seen in newborns from patients with IUI [31, 32].

The major limitation of any study involving the infectious etiology of preterm birth is the question of whether the infection preceded or was merely a result of PTL. We utilized the most widely accepted methods in the literature to identify IUI: AF culture and placental histology. Infection-related prematurity is likely a continuum of ascending infection from the lower tract to the upper tract, with the end stage being a positive amniotic fluid culture. Our findings of increased defensin levels in those with positive AF cultures as well as the correlation between defensin levels and placental pathology support this theory. Ideally, our patients with idiopathic PTL would have undergone delivery within 72 hours of presentation so that we could correlate defensin levels with presumed negative or low pathology scores. Because of the nature of clinical PTL this is impossible, but we recognize that patients with low pathology scores as well as mildly elevated defensin levels may have placental inflammation as a consequence rather than a cause of labor.

An important finding was that defensin levels of >2,500 ng/mL were detected in nine of 11 patients with placental pathology scores of >3, while levels were <2,500 ng/mL in all patients with scores of ≤3. Therefore, the degree of AF defensin level elevation may distinguish whether labor is most likely a cause or a result of infection.

Our data support labor and the antepartum period immediately prior to labor as contributing factors in the elevation of AF defensin levels. Neutrophil invasion near term is thought to be responsible for the process of cervical ripening [33]. This could explain the slightly increased defensin levels seen in patients undergoing amniocentesis for elective cesarean section at term.

Patients in labor at term have placental inflammation rates of 17% to 22% [6, 13, 34, 35], which in combination with normal neutrophil invasion of the cervix could account for the increased levels seen in the term laboring patients. Labor alone was not entirely responsible for the defensin level elevation, as patients with IUI had defensin levels fourfold higher than those of term laboring patients ($P < .03$). Furthermore, patients with IUI and a positive AF culture had defensin levels 50-fold higher than those of term laboring patients, while culture-negative patients had levels only twofold higher. Taken together, our data support the theory that IUI, with subsequent neutrophil recruitment and activation and subsequent release of granule products such as defensins, is both a cause and (to a lesser degree) a consequence of PTL.

In conclusion, AF defensin levels appear to be an excellent marker of subclinical IUI. Future work will focus on the correlation of AF defensin levels with defensin levels in specimens from less-invasive-testing sites such as cervicovaginal secretions and/or blood. The measurement of defensins in these specimens may ultimately help guide the management of preterm labor.

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