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The Role of Granulocyte Colony-Stimulating Factor in the Neutrophilia Observed in the Fetal Inflammatory Response Syndrome

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Abstract

OBJECTIVE—Fetal neutrophilia is present in two-thirds of cases with the fetal inflammatory response syndrome (FIRS). The mechanisms responsible for this finding have not been elucidated. Granulocyte Colony-Stimulating Factor (G-CSF) is the primary physiologic regulator of neutrophil production and plays a key role in the rapid generation and release of neutrophils in stressful conditions (i.e., infection). The objective of this study was to determine: 1) whether FIRS was associated with changes in fetal plasma G-CSF concentrations; and 2) if fetal plasma G-CSF concentrations correlated with fetal neutrophil counts, chorioamnionitis, neonatal morbidity/mortality and cordocentesis-to-delivery interval.

STUDY DESIGN—Percutaneous umbilical cord blood sampling was performed in a population of patients with preterm labor (n=107). A fetal plasma interleukin-6 (IL-6) concentration >11 pg/mL was used to define FIRS. Cord blood G-CSF was measured by a sensitive and specific immunoassay. An absolute neutrophil count was determined and corrected for gestational age. Receiver operating characteristic (ROC) curve, survival analysis and Cox proportional hazard model were employed.

RESULTS—1) G-CSF was detected in all fetal blood samples; 2) fetuses with FIRS had a higher median fetal plasma G-CSF concentration than those without FIRS ($p<0.001$); 3) a fetal plasma G-CSF concentration ≥134 pg/mL (derived from an ROC curve) was associated with a shorter cordocentesis-to-delivery interval, a higher frequency of chorioamnionitis (clinical and histological), intra-amniotic infection, and composite neonatal morbidity/mortality than a fetal plasma concentration below this cut-off; and 4) a fetal plasma G-CSF concentration ≥134 pg/mL was associated with a shorter cordocentesis-to-delivery interval (hazard ratio 3.2; 95% confidence interval 1.8-5.8) after adjusting for confounders.

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CONCLUSIONS—1) G-CSF concentrations are higher in the peripheral blood of fetuses with FIRS than in fetuses without FIRS; and 2) a subset of fetuses with FIRS with elevated fetal plasma G-CSF concentrations are associated with neutrophilia, a shorter procedure-to-delivery interval, chorioamnionitis and increased perinatal morbidity and mortality.

Keywords

G-CSF; FIRS; interleukin-6; pregnancy; preterm labor; fetal plasma; cordocentesis

INTRODUCTION

The fetal inflammatory response syndrome (FIRS), originally described in pregnancies complicated by spontaneous preterm labor and preterm prelabor rupture of membranes (PROM), is operationally defined as a fetal plasma interleukin-6 (IL-6) concentration of >11 pg/mL [1,2]. Soluble tumor necrosis factor receptor- 1 and -2, IL- 1β, IL-8, and CRP are also associated with FIRS [3-5]. Funisitis and chorionic vasculitis are considered the histological counterpart of FIRS [6,7]. A solid body of evidence supports the view that intra-amniotic infection/inflammation are considered to play a major role in the patophysiology of FIRS [8-12]. Fetuses with FIRS had a higher rate of neonatal morbidity including respiratory distress syndrome (RDS) [1,13], suspected or proven neonatal sepsis [1,13], pneumonia [1], bronchopulmonary dysplasia [14-16], necrotizing enterocolitis [1], intraventricular hemorrhage [1], periventricular leucomalacia as well as cerebral palsy [17-26] and a shorter cordocentesis-to-delivery interval in patients presenting with preterm PROM than those without FIRS [27]. Therefore, intra-amniotic infection/inflammation, a condition with an elevation of pro-inflammatory cytokines [28-41], caspase-1 (a component of inflammasome) [42], anti-inflammatory cytokines [43], chemokines [44-50], proteases/anti-proteases [51] , angiogenic factors [52], matrix-metalloproteinase [53-61], coagulation factors [62-65], adipocytokines [66-70], anti-microbial peptides [71,72], and prostaglandins [73,74] in amniotic fluid, is associated with neonatal morbidity/mortality among very preterm neonates [75-85].

Granulocyte colony-stimulating factor (G-CSF) is a cytokine produced mainly by monocytes and macrophages [86,87]. The production of the G-CSF is highly regulated and not constitutive [86]. In healthy adults, the circulating concentrations of G-CSF are usually below the limits of detection, and when detectable, are generally <100 pg/mL. During infection, however, G-CSF concentrations increase dramatically and may exceed 2000 pg/mL [88]. The biological activities of G-CSF include stimulation of neutrophil progenitors resulting in clonal expansion, increasing the bone marrow neutrophil storage pool [89] as well as the neutrophil count in peripheral blood [90], and improvement of mature neutrophil functions (e.g. phagocytosis and oxidative burst) [91]. Collectively, G-CSF is considered to act as a physiologic regulator and an emergency signal to increase neutrophil production/function under stressful conditions or infection [86].

Although septic preterm neonates tend to develop neutropenia [92], two-thirds of fetuses with FIRS have fetal neutrophilia [93]. The mechanisms responsible for fetal neutrophilia in FIRS have not been elucidated. The objective of this study was to determine: 1) whether FIRS was associated with changes in fetal plasma G-CSF concentrations; and 2) if fetal plasma G-CSF concentrations correlated with fetal neutrophil counts, clinical or histologic chorioamnionitis, neonatal morbidity/mortality and cordocentesis-to-delivery interval.

PATIENTS AND METHODS

Patients and eligibility

This retrospective cross-sectional study included singleton pregnancies with spontaneous preterm labor and intact membranes who were admitted to Hutzel Women's Hospital between March 1992 and June 1995. Patients were offered amniocentesis for the diagnosis of microbial invasion of the amniotic cavity and the assessment of fetal lung maturity. Patients who consented to amniocentesis were asked to participate in a research protocol that included cordocentesis to assess the fetal status. Exclusion criteria were multiple gestations, clinical signs of chorioamnionitis, vaginal bleeding, fetal distress, and unavailability of the fetal plasma samples for this study.

Clinical definition

Spontaneous preterm labor was diagnosed in the presence of regular uterine contractions (at least 3 in 30 minutes) and documented cervical change in patients with a gestational age between 20 and 36 6/7 weeks [1]. FIRS was defined as a fetal plasma IL-6 concentration greater than 11 pg/mL [1]. Intra-amniotic infection was defined as a positive microbiological culture of amniotic fluid. Clinical chorioamnionitis was diagnosed in the presence of a temperature elevation to 37.8°C or higher and two or more of the following criteria: uterine tenderness, malodorous vaginal discharge, fetal tachycardia >160 beats/min, and maternal leukocytosis >15,000 cells/mm^3 [94]. Composite neonatal morbidity/mortality was defined as the presence of any of the following conditions: respiratory distress syndrome (RDS) suspected or proved neonatal sepsis, pneumonia, bronchopulmonary dysplasia, intraventricular hemorrhage, necrotizing enterocolitis and perinatal death. The definitions of these neonatal complications have previously been described in detail [1]. The corrected neutrophil count was calculated from the ratio between the observed fetal neutrophil count and the expected mean fetal neutrophil count [95] according to gestational age at cordocentesis.

All patients provided written informed consent prior to the collection of samples. The collection and utilization of samples for research was approved by the Human Investigation Committee of Wayne State University, (Detroit, MI) and the Institutional Review Board of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD/NIH/DHHS). Many of these samples have been used in previous studies.

Clinical procedures and assays

All patients had a detailed ultrasonographic examination before amniocentesis and cordocentesis were performed. Electronic fetal monitoring was performed before and after the procedure to evaluate fetal well-being. Amniocentesis and cordocentesis procedures were performed with the freehand technique under ultrasound guidance. A 22-gauge needle was used, and a path was chosen for needle insertion that allowed the amniocentesis and cordocentesis procedures to be carried out with a single percutaneous needle insertion in approximately 95% of patients. Amniotic fluid studies included Gram stain, microbial cultures for aerobic and anaerobic bacteria as well as genital mycoplasmas, and the lecithin/sphyngomyelin ratio. The results of these tests were used for subsequent clinical management decisions. Fetal cord blood was collected into ethylenediaminetetra-acetic acid (EDTA) tubes. Kleihauer-Betke stains were performed on fetal blood, and all specimens were found to be free of maternal blood. Fetal blood was analyzed and complete white blood cell, platelet, and differential cell counts were performed. Results were made available for clinical management.

Plasma G-CSF and IL-6 concentrations were determined with commercially available enzyme-linked immunoassays obtained from R&D Systems (Minneapolis, MN). Briefly, the immunoassay utilized the quantitative sandwich technique and analyte concentrations were determined by interpolation from the standard curves. The inter- and intra-assay coefficients of variation for G-CSF were 4.8 % and 2.9 %, respectively, and those for IL-6 were 8.3% and 3.3% respectively. The sensitivities of the assays for G-CSF and IL-6 were 0.99 pg/mL and 0.06 pg/mL, respectively. The results of the analytes reported herein were not available for clinical decision-making. The presence or absence of acute inflammatory lesions in the extra-placental membranes (histologic chorioamnionitis) was assessed as previously described [7].

Statistical analysis

The Kolmogorov-Smirnov or Shapiro-Wilk test was used to determine if the data was normally distributed. A two-tailed Mann-Whitney U test was used to compare continuous nonparametric variables. Comparisons between proportions were performed using Chi-square or Fisher's exact tests. Correlation between two continuous variables was determined using Spearman's rank correlation test. Receiver operating characteristic (ROC) curve analysis was employed to determine a fetal plasma G-CSF concentration that identified patients who subsequently delivered within 7 days after cordocentesis. Kaplan-Meier survival analysis with a log rank test and Cox proportional hazard model were applied to examine the interval from cordocentesis-to-delivery according to fetal plasma G-CSF concentrations, while adjusting for confounding factors. A p-value <0.05 was considered statistically significant. Analysis was performed with SPSS, version 12 (SPSS Inc., Chicago, IL, USA).

RESULTS

Demographic and clinical characteristics

This study included 107 women who presented with spontaneous preterm labor and intact membranes, 23% (25/107) of which had a diagnosis of FIRS. Seventy-six patients (71%) delivered at less than 37 weeks of gestation. There were 2 perinatal deaths, and in both cases, the infants were delivered before 24 weeks of gestation. Intra-amniotic infection was diagnosed in 11.2% (12/107) of cases. The organisms were *Ureaplasma urealyticum* (n=7), *Fusobacterium* species (n=3), *Pseudomonas aeruginosa* (n=1) and *Corynebacterium jeikeium* (n=1). As expected, patients with FIRS had a significantly higher rate of clinical chorioamnionitis, microbiological proven intra-amniotic infection, histologic chorioamnionitis, composite neonatal morbidity/mortality and a shorter cordocentesis-to-delivery interval than those without FIRS (all p<0.05; see Table I).

Neutrophil count and fetal plasma G-CSF concentration in fetuses with FIRS

Immunoreactive G-CSF was detected in all samples of fetal blood. Fetuses with FIRS had a median absolute neutrophil count, corrected neutrophil count and plasma G-CSF concentration higher than those without FIRS (all p<0.05; see Table I and Figure 1). Among patients with FIRS, the fetal plasma G-CSF concentration was correlated with fetal plasma IL-6 concentration (Spearman's rho = 0.5; p=0.02; Figure 2a) and the corrected fetal neutrophil count (Spearman's rho = 0.6; p=0.007; Figure 2b). The fetal plasma G-CSF concentration had an inverse correlation with gestational age at cordocentesis (Spearman's rho = −0.5; p=0.02) and birthweight (Spearman's rho = −0.7; p<0.001). In contrast, among patients who subsequently delivered at term (n=31), there was a significant relationship between fetal plasma G-CSF concentration and gestational age at cordocentesis (Spearman's rho = 0.6; p=0.001, especially between 27 and 35 weeks, Figure 2c) as well as fetal neutrophil count (Spearman's rho = 0.8; p<0.001, Figure 2d). The median fetal plasma G-

CSF concentration of women who presented with spontaneous preterm labor between 21 and 35 weeks and subsequently delivered at term was 45.3 pg/mL and ranged between 8.0 and 134.0 pg/mL.

Elevated fetal plasma G-CSF concentration is associated with a shorter cordocentesis-to-delivery interval and a higher frequency of chorioamnionitis

The prevalence of patients who delivered within 7 days after cordocentesis was 32% (34/107). ROC curve analysis indicated that a fetal plasma G-CSF concentration ≥134 pg/mL had an area under the curve of 0.85 (95% CI 0.76-0.92; p<0.001), a sensitivity of 68% (23/34), a specificity of 92% (67/73), a positive predictive value of 79% (23/29), and a negative predictive value of 86% (67/78) for the identification of patients who subsequently delivered within 7 days. The corresponding indices for fetal plasma IL-6 concentration >11 pg/mL were 0.72 (0.60-0.83), 52.9%, 90.4%, 72% and 80.5%, respectively (see Figure 3).

Women with a fetal plasma G-CSF concentration ≥134 pg/mL had a higher frequency of clinical chorioamnionitis (17.2% vs. 2.6%; p=0.02), intra-amniotic infection (37.9% vs. 1.3%; p<0.001), histologic chorioamnionitis (63.6% vs. 18.8%; p=0.001) and composite neonatal morbidity/mortality (69% vs. 33.3%; p<0.001; see Table II) than those with a fetal plasma G-SCF concentration below this cutoff.

Fetal plasma G-CSF concentration: an independent predictor of procedure-to-delivery interval

To assess the relationship between fetal plasma G-CSF concentration and the duration of the cordocentesis-to-delivery interval, Kaplan-Meier survival analysis was employed. Spontaneous labor and delivery was considered the event of interest, and the cordocentesis-to-delivery interval of patients who were delivered for fetal or maternal indications was treated as censored observations (a censoring time equal to the cordocentesis-to-delivery interval).

The median cordocentesis-to-delivery interval in patients with a fetal plasma G-CSF concentration ≥134 pg/mL was significantly shorter than that of those with a fetal plasma G-CSF concentration below this cutoff value [G-CSF >134 pg/mL; median procedure-to-delivery interval 1.75 days, interquartile (IQR) 1-6.4 days, censored 5 out of 29 events vs. G-CSF <134 pg/mL; median procedure-to-delivery interval 33 days, IQR range 18-55 days, censored 12 out of 78 events; p<0.0001; see Figure 4). Cox proportional hazard modeling was used to examine the relationship between the duration of the cordocentesis-to-delivery interval and fetal plasma G-CSF concentrations while adjusting for cervical dilatation at admission, the status of amniotic fluid culture, gestational age at cordocentesis, the presence of clinical chorioamnionitis, and the exposure to antenatal steroids or tocolysis prior to cordocentesis. Only fetal plasma G-CSF concentration ≥134 pg/mL and cervical dilatation were independent predictors of cordocentesis-to-delivery interval with a hazard ratio of 3.2 (95% CI 1.8-5.8) and 1.6 (95% CI 1.3-1.9), respectively (Table III).

A subset of FIRS has a high fetal plasma G-CSF concentration

Only 72% (18/25) of fetuses with FIRS had a high fetal plasma G-CSF concentration, while 62% (18/29) of fetuses with a high fetal plasma G-CSF concentration had a diagnosis of FIRS. Patients who had both fetal plasma G-CSF concentrations >134 pg/mL and IL-6 concentrations >11 pg/mL had a significantly shorter procedure-to-delivery interval (median 1.1 day, IQR 0.4-1.8 days) than those who had either isolated fetal plasma IL-6 concentrations >11 pg/mL (median 26 days, IQR 2-46 days; p=0.01) or fetal plasma G-CSF concentrations >134 pg/mL alone (median 3.5 days, IQR 0.8-20.2 days; p=0.03) (Table IV). There was no significant difference in the cordocentesis-to-delivery interval between those

who had fetal plasma IL-6 concentrations >11 pg/mL and those who had high fetal plasma G-SCF concentrations alone (p=0.2). The frequency of intra-amniotic infection was the highest in a subset of patients with an elevation of both cytokines in fetal plasma [high for both cytokines 50% (9/18) vs. high for G-CSF alone 18.2% (2/11), high for IL-6 alone 0% (0/7) and low for both cytokines 1.4% (1/71); Chi-square for trend: p<0.001, see Table IV).

DISCUSSION

Principal findings of the study

1) Fetuses with FIRS had a higher median plasma G-CSF concentration than those without FIRS; 2) patients with spontaneous preterm labor with intact membranes and a fetal plasma G-CSF concentration >134 pg/mL had a shorter cordocentesis-to-delivery interval, a higher frequency of chorioamnionitis (clinical and histological) and microbiologically proven intra-amniotic infection and composite neonatal morbidity/mortality than those with a fetal plasma G-CSF concentration below this cutoff; 3) fetal plasma G-CSF concentration was an independent predictor of cordocentesis-to-delivery interval after adjustment for other confounders; 4) a subset of patients with FIRS who had elevated plasma G-CSF concentrations had a significantly shorter procedure-to-delivery interval and higher frequency of intra-amniotic infection than those who had either isolated high fetal plasma IL-6 or high G-CSF concentration alone; 5) among patients with FIRS, the fetal plasma G-CSF concentration correlated with fetal plasma IL-6 concentration and with corrected fetal neutrophil count; and 6) in the group of women with an episode of spontaneous preterm labor who delivered at term, there was a significant relationship between gestational age and fetal G-CSF concentration as well as between fetal plasma G-CSF concentration and absolute fetal plasma neutrophil count.

Physiologic roles of G-CSF

Monocytes and macrophages are the primary sources of G-CSF [86,96,97], although this hematopoietic growth factor can be produced by endothelial cells [98], fibroblasts [99] and mesothelial cells [100]. Monocytes and macrophages can be stimulated to produce G-CSF upon incubation with lipopolysaccharides (LPS), IL-1, IL-3, IL-4, interferon gamma and granulocyte-macrophage colony-stimulating factor [101]. G-CSF exerts its biological activities through binding to a specific G-CSF receptor which has been located on both hematopoietic cells (such as myeloid progenitors, mature granulocytes, platelets, monocytes, lymphocytes) [102-105] and non-hematopoietic cells (such as vascular endothelial cells [106], cardiac myocytes [107], intestinal villi [108], lung [109], kidney [110], skeletal muscle [110], neural stem cells [111] and syncytiotrophoblast [112,113]). G-CSF plays a key role in the rapid generation and release of neutrophils from the storage pool in stressful conditions such as infection [101,113,114]. Moreover, G-CSF not only attracts neutrophils [115], but also improves neutrophil function by increasing phagocytosis, superoxide release, and delays neutrophil apoptosis [116].

Conflicting results have been reported on the change of G-CSF concentrations in the umbilical cord blood of preterm neonates as a function of gestational age. Either an increase [90,117-119], a decrease [120,121] or no change [122,123] in the circulating G-CSF concentration with gestational age has been reported. In this study, we demonstrate for the first time that fetal plasma G-CSF concentrations increased as a function of gestational age (Spearman Rho = 0.6) in patients presenting with preterm labor who delivered at term. Moreover, plasma G-CSF concentration strongly correlated with the absolute fetal neutrophil count (Spearman Rho = 0.8) suggesting that G-CSF is a physiologic regulator of neutrophil count in peripheral blood of preterm fetuses.

The roles of G-CSF in growth and maturation beyond hematopoiesis remain unknown. Recent animal experiments suggested that G-CSF is an essential neurotrophic factor which plays a role in the proliferation, differentiation and functional integration of neural cells in the hippocampus, an area important for memory formation and development of motor skills [124,125]. Moreover, the recombinant form of G-CSF may have a beneficial role in necrotizing enterocolitis [126], ischemic heart disease and hypoxic-ischemic brain injury [111,127-129] by inhibiting apoptosis, promoting the local neural stem cells [130] and recruiting bone marrow-derived stem cells to the sites of injury [131].

Fetuses with FIRS have a higher plasma G-CSF concentration than those without FIRS

Accumulating evidence suggests that fetuses with FIRS have evidence of multi-organ involvement as identified by biochemical and biophysical changes observed in the adrenal gland [132], heart [133], lung [14], brain [18,19,24,134], skin [135] and hematopoietic systems [93,136]. The latter includes an elevation of neutrophil count [93] as well as phenotypic evidence of monocyte-neutrophil activation in patients who delivered within 72 hours of cordocentesis [136]. Moreover, umbilical cord blood from patients with acute funisitis, the histological counterpart of FIRS [7], had phenotypic [higher median mean channel brightness of CD14, CD64, and CD66b on granulocytes and of CD64 on monocytes] and metabolic changes (increased basal intracellular reactive oxygen species and oxidative burst in monocytes) on leukocytes consistent with activation of the fetal innate immune response[137]. Indeed, microarray analysis of leukocyte RNA revealed differential expression of 541 unique genes. Moreover, ontological and pathway analyses yielded significant enrichment of biological processes including antigen processing and presentation, immune response, and processes critical to cellular metabolism[138]. The increased plasma G-CSF concentration in fetuses with FIRS and the relationship between the fetal plasma G-CSF concentrations and the corrected neutrophil count observed in the current study could, at least in part, explain the quantitative and qualitative changes in neutrophils of fetuses with FIRS.

Is an increased fetal plasma G-CSF concentration beneficial or detrimental to the fetus?

Experimental G-CSF knock-out mice are viable, fertile, and apparently healthy, but they have a chronic neutropenia and markedly impaired ability to control bacterial infection [139]. These observations suggest that the function of G-CSF is beneficial to maintain the normal quantitative physiologic balance of neutrophil production and is an emergency granulopoietic signal against infections. Several studies in newborn animals with experimental sepsis [140] and in human neonates with sepsis indicate that the administration of recombinant G-CSF increases the neutrophil count [141], improves neutrophil function [142] and might reduce sepsis-related mortality [143]. However, a concern is the potential detrimental effects of recombinant G-CSF treatment in aggravating acute lung injury in neonates with RDS [144,145].

The most recent Cochrane review concluded that there is insufficient evidence to support the introduction of recombinant G-CSF either as a treatment of established infection to reduce mortality or as a prophylaxis to prevent systemic infection in high-risk neonates [146]. However, this subject should be further investigated since there is evidence that pharmacologic G-CSF treatment may reduce mortality in a subset of neonates with severe neutropenia [146,147].

Elevated fetal plasma G-CSF concentration is associated with a shorter cordocentesis-to-delivery interval, chorioamnionitis and neonatal morbidity/mortality

Immunoreactive G-CSF has been detected in amniotic fluid, neonatal urine and tracheobronchial secretion as well as umbilical cord, neonatal and maternal blood. Patients

who delivered preterm, especially those with detectable endotoxin in the amniotic fluid and those with clinical or histologic chorioamnionitis, had a median amniotic fluid concentration of G-CSF higher than those who delivered at term or those without clinical/histologic chorioamnionitis [148-151]. Women in labor at term had a higher mean amniotic fluid concentration of G-CSF than those without labor [148]. Similarly, neonates born from mothers with clinical chorioamnionitis and those with signs of infection had a higher mean G-CSF concentration in serum, urine and tracheobronchial secretion than those who were born from mothers without clinical chorioamnionitis or signs of infection [118,149,150]. Consistent with these findings, the current study demonstrated that elevated fetal plasma G-CSF concentration was associated with clinical/histological chorioamnionitis, intra-amniotic infection and composite neonatal morbidity/mortality.

Elevated maternal serum G-CSF concentration has been reported in patients with clinical chorioamnionitis [149]. In asymptomatic women at 24 and 28 weeks of gestation, an elevation of this cytokine is associated with subsequent early spontaneous preterm delivery (<32 weeks), but not with late preterm birth [152]. The authors also noted that spontaneous preterm delivery often occurs within 4 weeks after the blood sampling in such cases [152]. However, a recent study in asymptomatic women between 6 and 18 weeks of gestation reported an association between elevated maternal serum G-CSF concentrations and spontaneous preterm birth with an odds ratio of 1.52 (for each one standard deviation increase) [153]. Consistent with these observations, in the current study, fetal plasma G-CSF concentration is an independent predictor of cordocentesis-to-delivery interval after adjustment for other potential confounders. Although dexamethasone has been shown to increase the production of G-CSF in mononuclear cells of term and preterm infants [154], a fetal plasma G-CSF concentration >134 pg/mL is associated with a shorter cordocentesis-to-delivery interval after adjustment for the exposure of antenatal steroids.

The increased fetal plasma G-CSF concentration observed herein is unlikely to result mainly from transplacental passage from the mother's circulation since the G-CSF concentration in different fetal compartments (amniotic fluid, umbilical cord blood or neonatal serum: mean 5,520 pg/mL, 8,281 pg/mL and 4,364 pg/mL, respectively) is much higher than that observed in maternal blood (mean 186 pg/mL) during clinical chorioamnionitis [149]. Despite the relative low transplacental passage of G-CSF, a randomized clinical trial demonstrated that the administration of recombinant G-CSF to women during preterm labor could increase neutrophil proliferative pool in the neonatal bone marrow after preterm delivery [155].

FIRS may have multiple etiologies—The original definition of FIRS was described in fetuses with preterm labor and preterm PROM, and was often associated with microbial invasion of the amniotic cavity [1]. To date, FIRS has been largely observed in pregnancy complications involving infection. However, similar to systemic inflammatory response syndrome in adults [156], the fetus might also be able to mount an inflammatory response to non-microbial-related insults (eg: fetal anemia due to Rh alloimmunization) [157]. In this study, only a subset of fetuses with FIRS had an elevation of plasma G-CSF concentrations. This subgroup of patients had a shorter procedure-to-delivery interval, and had a higher rate of a positive microbial culture in amniotic fluid than those with FIRS without G-CSF elevation. It is possible that FIRS accompanied by an elevation of G-CSF concentration is a consequence of bacterial or fungal-related insults [139,158,159]. Consistent with this view, plasma G-CSF concentrations have been proposed to be an early marker of proven bacterial or fungal infection in neonates undergoing an evaluation for sepsis [122]. The possibility that a subset of fetuses with FIRS who presents with systemic inflammatory response (elevation of IL-6) is a consequence of non-bacterial or non-fungal infection (e.g., fetal

anemia ue to Rh alloimmunization [157], viral infection or other non-infectious etiologies) should be considered.

Strength and limitations of the study

This study is the first description of plasma G-CSF concentration ue to Rh alloimmunization [157] in preterm fetuses with FIRS and those who subsequently delivered at term. We consider patients who presented with preterm labor without intra-amniotic infection and subsequently delivered at term be the closest to normal fetuses that could have cordocentesis performed, since this procedure could not have been performed in pregnant women without a clinical indication. Previous studies of the changes in plasma G-CSF concentrations as a function of gestational age of "normal" preterm neonates may be inaccurate for the following reasons: 1) preterm neonates are not "normal" by definition (they have to be delivered for maternal or fetal indications); 2) the difficulty in the accurate assessment of infection in premature infants; and 3) the high rate of subclinical intrauterine infection in patients with early preterm delivery.

In conclusion, our observations suggest that G-CSF is detectable in the plasma of preterm fetuses. The fetal plasma G-CSF concentration is higher in fetuses with FIRS than in those without FIRS. The elevation of G-CSF correlates with the corrected fetal neutrophil counts suggesting that G-CSF is responsible for fetal neutrophilia in FIRS. A subset of fetuses with FIRS, who had a fetal plasma G-CSF concentration ≥134 pg/mL had a higher frequency of chorioamnionitis, composite neonatal morbidity/mortality, and a shorter cordocentesis-to-delivery interval than those with a fetal plasma G-CSF concentration below this cut-off.

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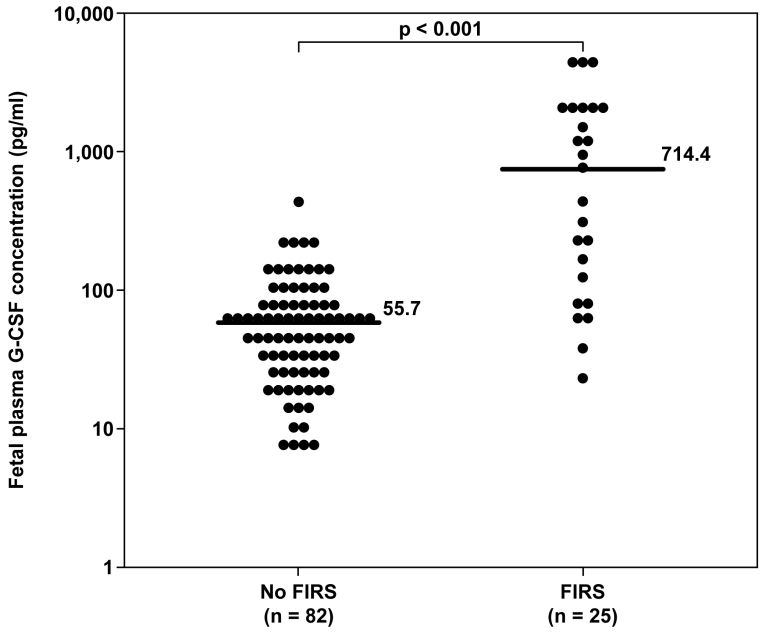

Figure 1. Comparison of the fetal plasma granulocyte-colony stimulating factor (G-CSF) concentration between fetuses with FIRS and those without FIRS

Fetuses with FIRS had a median fetal plasma concentration of G-CSF higher than those without FIRS [FIRS: median 714.4 pg/mL, interquartile range (IQR) 104-1918 pg/mL vs. without FIRS: median 55.7 pg/mL, IQR 30.1-86.1 pg/mL; p<0.001]. The y-axis is depicted in log scale.

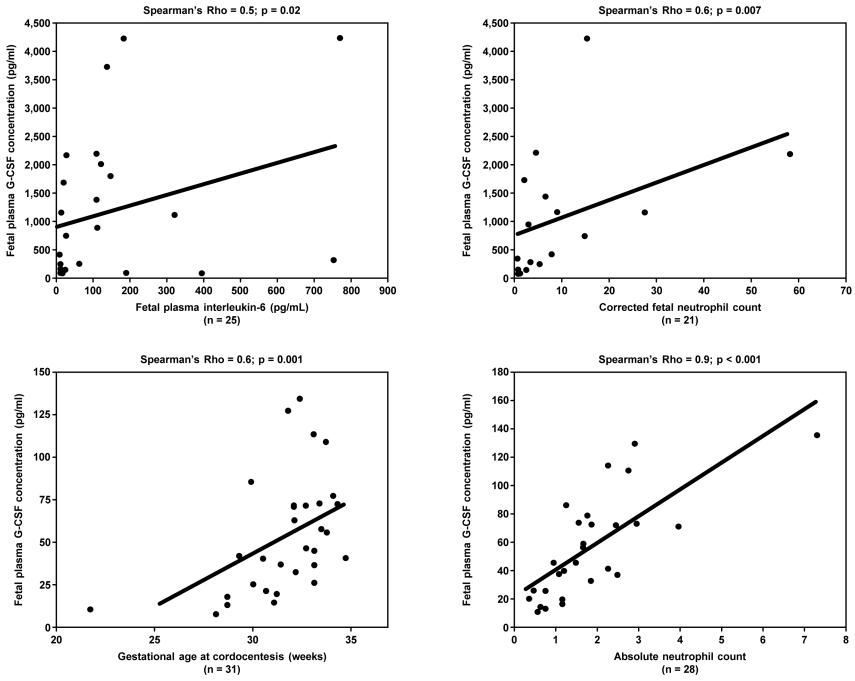

Figure 2. Relationships between fetal plasma G-CSF concentration and other variables

Among patients with FIRS, fetal plasma G-CSF concentrations correlated with fetal plasma IL-6 concentration (Spearman's rho = 0.5; p=0.02; Figure 2a) and corrected fetal neutrophil count (Spearman's rho = 0.6; p=0.007; Figure 2b). In contrast, among patients who subsequently delivered at ≥37 weeks, there was a significant relationship between fetal plasma G-CSF concentration and gestational age at cordocentesis (Spearman's rho = 0.6; p=0.001; Figure 2c) as well as absolute fetal neutrophil count (Spearman's rho = 0.8; p<0.001; Figure 2d).

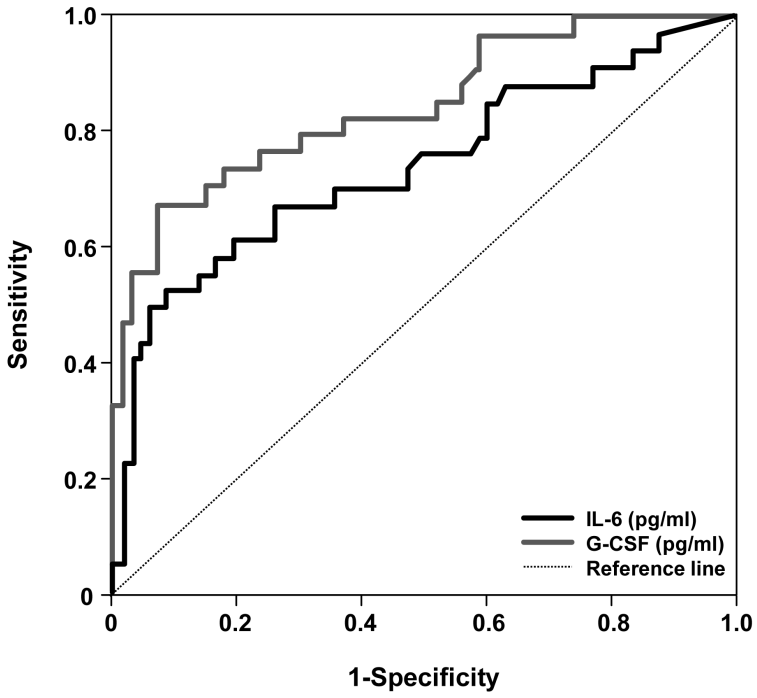

Figure 3. Receiver operating characteristic (ROC) curve analysis for the identification of patients who subsequently delivered within 7 days after cordocentesis

A fetal plasma G-CSF concentration ≥134 pg/mL (grey line) and a fetal plasma IL-6 concentration >11 pg/mL (black line) had an area under the curve of 0.85 (95% CI 0.76-0.92; p<0.001) and 0.72 (95% CI 0.60-0.83; p<0.001), respectively, for the identification of patients who subsequently delivered within 7 days.

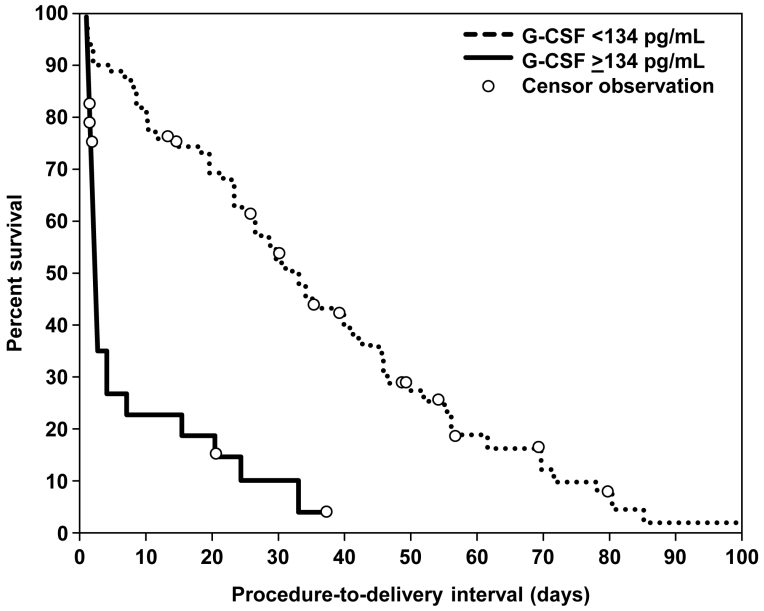

Figure 4. Survival curves for cordocentesis-to-delivery interval according to fetal plasma concentrations of G-CSF

Spontaneous labor and delivery was entered in the analysis as the event of interest. Patients who delivered by fetal or maternal indications were censored. Women with fetal plasma concentrations of G-CSF >134 pg/mL had a significantly shorter procedure-to-delivery interval than those with a fetal plasma G-CSF concentration below this cutoff value: [G-CSF >134 pg/mL; median procedure-to-delivery interval 1.75 days, interquartile (IQR) 1-6.4 days, censored 5 out of 29 events vs. G-CSF <134 pg/mL; median procedure-to-delivery interval 33 days, IQR range 18-55 days, censored 12 out of 78 events; log rank test $p<0.0001$].

Table I

Clinical characteristics of the study population

	No FIRS n = 82	FIRS n=25	p
Age (years)	22.0 (20.0-27.0)	23 (19.0-25.5)	0.6
GA at admission (weeks)	32.0 (29.1-33.0)	29.0 (25.1-31.3)	0.003
Interval to delivery (days)	27.0 (9.9-46.0)	1.6 (0.5-13.9)	<0.001
Preterm delivery	53 (64.6%)	23 (92%)	0.01
GA at delivery (weeks)	35.8 (33.6-38.0)	29.4 (25.1-35.0)	<0.001
Birthweight (grams)	2,608 (2,097-3,042)μ	1,230 (740-2,622)	<0.001
Clinical chorioamnionitis	2 (2.4%)	5 (20%)	0.007
Positive amniotic fluid culture	3 (3.7%)	9 (36.0%)	<0.001
Delivery within 24 hours	7 (8.5%)	8 (32%)	0.003
Delivery within 48 hours	8 (9.8%)	15 (60%)	<0.001
Delivery within 7 days	16 (19.5%)	18 (72%)	<0.001
Histologic chorioamnionitis	8 (22.9%) γ	12 (63.2%)π	0.003
Composite neonatal morbidity	27 (32.9%)	19 (76%)	<0.001
Fetal interleukin-6 (pg/ml)	5.2 (3.2-6.7)	69.1 (18.4-169.4)	<0.001
Fetal neutrophil count ($x10^9$/L)	1.5 (0.7-2.4)α	2.5 (1.4-3.9)β	0.028
Corrected fetal neutrophil count	1.8 (1.3-2.5)α	3.4 (1.1-8.9)β	0.028
Fetal G-CSF (pg/ml)	55.7 (30.1-86.1)	714.4 (104.3-1918.7)	<0.001

FIRS: Fetal Inflammatory Response Syndrome

Values are expressed as median (interquartile range) or number (percent)

GA: gestational age; G-CSF: granulocyte colony stimulating factor

μ: n=81; γ: n=35; π: n=19; α: n=75; β: n=21

Table II

Clinical characteristics of the study population according to fetal plasma concentrations of G-CSF ≥134 or < 134 pg/ml

	G-CSF <134 pg/ml (n=78)	G-CSF ≥ 134 pg/ml (n=29)	p
Age (years)	22.0 (19.8-27)	23.0 (19-25.5)	0.7
GA at cordocentesis (weeks)	31.8 (28.9-33.0)	30.5 (25.4-32.3)	<0.001
Cervical dilatation (cm)	1.5 (0.9-2.5)	2.5 (1.0-4.0)γ	0.009
Interval to delivery (days)	29.9 (13.6-48.7)	1.6 (0.5-4.9)	<0.001
Preterm delivery	48 (61.5%)	28 (96.6%)	<0.001
GA at delivery (weeks)	36.1 (34.0-38.0)	30.5 (25.5-34.7)	<0.001
Birthweight (grams)	2,640 (2,200-3,104)μ	1680 (747-2,342)	<0.001
Clinical chorioamnionitis	2 (2.6%)	5 (17.2%)	0.02
Positive amniotic fluid culture	1 (1.3%)	11 (37.9%)	<0.001
Exposure to antenatal steroids prior to cordocentesis	19 (24.4%)	13 (44.8%)	0.04
Exposure to tocolysis prior to cordocentesis	68 (87.2%)	27 (93.1%)	0.5
Spontaneous delivery	66 (84.6%)	24 (82.8%)	0.8
Histologic chorioamnionitis	6 (18.8%) α	14 (63.6%) β	0.001
Composite neonatal morbidity	26 (33.3%)	20 (69.0%)	0.001

Values are expressed as median (interquartile range) or number (percent)

G-CSF: granulocyte colony stimulating factor; GA: gestational age;

Tocolysis: magnesium sulfate, indocin, or terbutaline

γ: n=28;μ: n=77; α: n=32; β: n=22

Table III

Hazard ratio for the procedure-to-delivery interval (days)*

Independent variables	Hazard ratio	95% Confidence Interval
Fetal plasma G-CSF ≥134 pg/ml	3.2	1.8-5.8
Cervical dilation (cm)	1.6	1.3-1.9
Gestational age at procedure (weeks)	1.1	0.9-1.15
Clinical chorioamnionitis	2.6	0.8-8.7
Exposure to antenatal steroids prior to cordocentesis	0.9	0.6-1.6
Exposure to tocolysis prior to cordocentesis	1.2	0.5-3.0
Positive amniotic fluid culture	1.2	0.5-2.9

* n=106 (one patient had no information on cervical dilatation)

Table IV

Procedure-to-delivery interval (days) and composite neonatal morbidity/mortality according to fetal plasma G-CSF and IL-6 concentrations

	Number (%)	GA at delivery (weeks)	Procedure-to-delivery interval (days)	Positive amniotic fluid culture
IL-6 ≤ 11 pg/ml G-CSF < 134 pg/ml	71 (66.4%)	36.1 (34.0-38.0)	30 (14-49)	1 (1.4%)
IL-6 > 11 pg/ml G-CSF < 134 pg/ml	7 (6.5%)	35.1 (34.8-37.9)	26 (2.0-46.0)	0
IL-6 ≤ 11 pg/ml G-CSF > 134 pg/ml	11 (10.3%)	33.6 (31.3-35.9)	3.5 (0.8- 20.2)	2 (18.2%)
IL-6 > 11 pg/ml G-CSF ≥ 134 pg/ml	18 (16.8%)	28.4 (24.4-31.1)	1.1 (0.4-1.8)	9 (50%)

Values are expressed as median (interquartile range) or number (percent)

GA: gestational age; G-CSF: granulocyte colony stimulating factor; IL: interleukin