

RESEARCH ARTICLE

Midkine and Pleiotrophin Concentrations in Amniotic Fluid in Healthy and Complicated Pregnancies

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Abstract

Background

Midkine (MDK) and pleiotrophin (PTN) are heparin-binding growth factors that, in rodents, are highly expressed in early life and decrease to undetectable levels by adulthood. The potential roles of MDK and PTN in human growth and development are not completely elucidated.

Method and Findings

To delineate the role of MDK and PTN in human development, we developed high sensitivity assays to measure their concentrations in amniotic fluid (AF) at various gestational ages in both healthy and complicated pregnancies. We found that both of these growth factors could be readily measured in AF and that the concentrations were higher than most cytokines previously reported in AF.

Conclusion

The concentration of MDK but not that of PTN declined with gestational age. Both MDK and PTN concentrations were found to be lower in pregnancies that were complicated by chorioamnionitis at term, raising the possibility that these growth factors might be useful as markers for infection.

Introduction

Amniotic fluid (AF) provides an essential, complex, and dynamic milieu for the growing fetus that changes with progression of the pregnancy [1]. AF contains nutrients (carbohydrates, proteins and peptides, and lipids), growth factors, and cytokines that facilitate fetal growth. The functions and significance of individual growth factors in human AF remain incompletely understood. Various cytokines, such as interleukin (IL)-6, IL-8, IL-10, IL-11, IL-15, tumor necrosis factor (TNF)- α , transforming growth factor (TGF)- β , and vascular endothelial growth factor (VEGF), have been studied as potential markers for a variety of conditions including pre-eclampsia, intrauterine growth retardation, preterm labor, preterm premature rupture of membranes (PPROM), and intra-amniotic inflammation/infection, but the variability in concentrations of AF cytokines often results in values that overlap with those during normal pregnancies, thus limiting the clinical utility of AF cytokine measurements [2–5].

Midkine (MDK) and pleiotrophin (PTN) are two closely-related heparin-binding growth factors that are rich in both basic amino acids (arginine, lysine, and histidine) and cysteines [6–7]. The biological activities of these growth factors include promotion of growth, cell migration, tissue morphogenesis, and chemokine expression in numerous target cell types [8–9]. In rodents, these growth factors are highly expressed in early life in multiple organs and decrease to low levels by adulthood [10–14]. The roles of MDK and PTN in human growth and development *in utero* are yet to be elucidated.

Neither MDK nor PTN concentrations have been assessed in human amniotic fluid. We therefore developed high sensitivity assays to measure the concentrations of both factors in human amniotic fluid, assessing the levels at various gestational ages, in both healthy pregnancies and pregnancies with a variety of common complications.

Materials and Methods

Study population

Specimens were obtained from the Biologic Tissue Bank of the Perinatology Research Branch (PRB) of the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) (ClinicalTrials.gov:NCT00340249). Plasma samples had been obtained in 1999–2001, and amniotic fluid 1990–2005. Clinical data were extracted from the associated Perinatal Database. All subjects provided written informed consent. The Institutional Review Board of the NICHD approved the use of clinical data and biological specimens. MDK was measured in maternal plasma from singleton complicated and healthy pregnancies ($n = 114$) and non-pregnant, healthy, age-matched controls ($n = 16$, Fig 1A). MDK ($n = 202$) and PTN ($n = 170$) were measured in AF from singleton pregnancies grouped according to outcome of pregnancy (Fig 1B and 1C). PTN was not measured in all samples due to insufficient sample volume.

Biological samples and analysis

Peripheral blood was collected in a glass tube containing citrate. The blood was centrifuged at 4°C for 15 min at 3,000 g within 2 h of venipuncture. Plasma was aliquotted in plastic tubes and stored at -80°C until MDK assay. AF not required for clinical assessment was centrifuged in glass tubes for 10 min at 4°C and stored at -70°C in plastic tubes until assay. The investigator performing the assays was blinded to all clinical data.

Clinical definitions

Gestational age (GA) was self-reported based on the last menstrual period and confirmed by ultrasound. In cases of inconsistency between reported last menses and sonographic

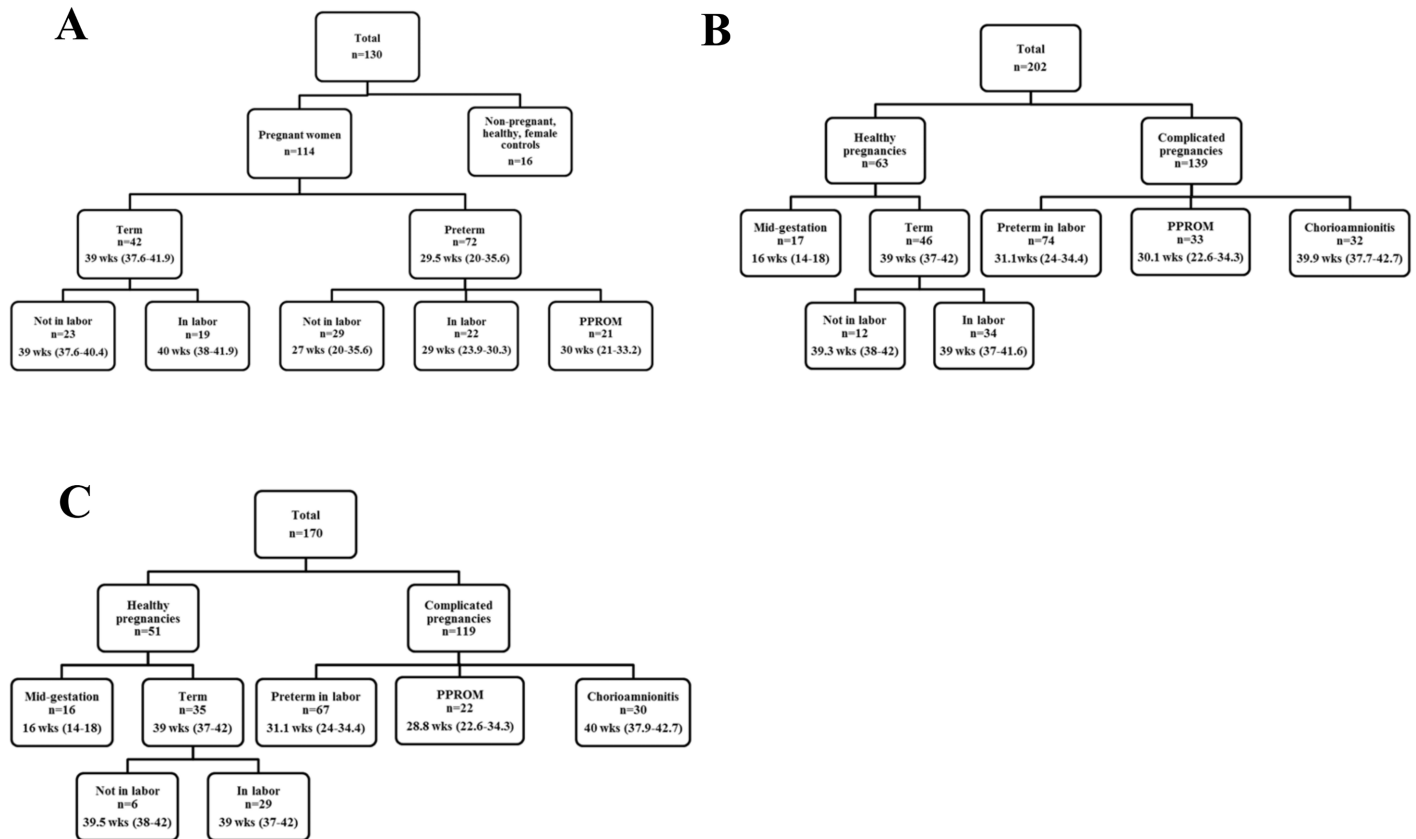


Fig 1. Flowchart of samples evaluated for plasma midkine (1A), amniotic fluid midkine (1B), and amniotic fluid pleiotrophin (1C). PPROM, preterm premature rupture of membranes; n, number of samples; gestational age represents the median (range) age at which sample was obtained.

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determination of GA, ultrasonographic age was used. Term gestation was defined by gestational age 37–42 weeks. Preterm premature rupture of membranes (PPROM) was diagnosed as accumulation of AF in the vagina before 37 weeks of gestation and confirmed by a positive nitrazine test and/or positive ferning test. Clinical chorioamnionitis was diagnosed when maternal temperature exceeded 37.8°C with the presence of at least 2 of the following criteria: uterine tenderness, malodorous vaginal discharge, maternal leukocytosis (>15,000 cells/mm³), maternal tachycardia (>100 beats/min), or fetal tachycardia (>160 beats/min) [15–16].

Midkine sandwich ELISA for plasma and amniotic fluid

MDK sandwich enzyme-linked immunosorbent assay (ELISA) was performed using a commercial kit (Biovendor, Czech Republic) with modifications as previously described [17]. In particular, poly-L-lysine was added to the biotin-labelled detection antibody solution provided with the kit to increase responsiveness of the ELISA. Prior to assay, 125 µL of plasma were diluted in 125 µL of TBSTA (TBS, 1% BSA, 0.5% Tween 20, pH 7.4) and 5 µL of AF were diluted in 245 µL of TBSTA. The rest of the procedure was identical to the procedure previously described [17]. The detection limit for plasma MDK was 8.7 pg/mL and for AF MDK was 7 pg/mL. Inter-assay CV was 3.2% for plasma and 11.7% for AF. The assay showed good parallelism in plasma (S1 Fig). The detection range of the assay was 0–0.8 ng/ml.

Pleiotrophin sandwich ELISA for amniotic fluid

A PTN sandwich ELISA was developed in our lab. The mouse anti-pleiotrophin monoclonal antibody (3B10, produced in the lab of Dr. Anton Wellstein) was diluted to 0.5 $\mu\text{g}/\text{mL}$ in PBS and 100 $\mu\text{L}/\text{well}$ was incubated in a 96-well plate at 4°C overnight. The wells were washed 3 times with 250 μL per well of PBST (PBS, 0.05% Tween 20). The wells were blocked with 250 μL per well of PBS containing 3% BSA and 0.2% Tween 20 for 2 hours at 4°C. Without washing, the plate was inverted and dried by tapping vigorously against a paper towel. Subsequently, 5 μL AF were diluted in 245 μL of PBSTA (PBS, 1% BSA, 0.5% Tween 20), and 100 μL of the diluted samples were pipetted in duplicate into the wells. The plate was incubated with gentle agitation at room temperature for 2 hours and then washed 3 times with 250 μL per well of PBST. After tapping the inverted plate to remove residual fluid, a biotinylated anti-human pleiotrophin goat IgG (R & D systems, USA) were added at a concentration of 500 ng/mL in 0.9% saline containing 5.7 meq/L calcium chloride and 0.5% BSA at pH 6. The plate was incubated with gentle agitation at room temperature for an hour. Then the wells were washed 5 times with 250 μL of PBST per well. After tapping, 100 μL of streptavidin-HRP conjugate solution (Thermo Scientific, USA) were added at a concentration of 25 ng/mL in PBS to each well and the plate was incubated at room temperature for 30 minutes with gentle agitation. After washing 5 times with PBST and tapping, 100 μL of TMB (eBioscience, USA) were added to each well. The plate was covered with aluminum foil and incubated for 7 minutes at room temperature. Color development was stopped by adding 100 μL of stop solution (0.16M sulfuric acid). The absorbance of each well was measured using a microplate reader set to 450 nm (Synergy 4, BioTek, USA). The detection limit for AF PTN was 10 pg/mL . The inter-assay CV was 2.7%. The detection range of the assay was 0–1.8 ng/mL .

Validation of AF MDK and PTN assays

To assess the specificity of the ELISAs, we took advantage of the fact that both MDK and PTN bind avidly to heparin. Both MDK and PTN were removed from AF using Heparin-Sepharose beads and this procedure essentially eliminated all ELISA signals for both MDK and PTN (Fig 2A and 2B). To further validate the MDK and PTN assays, AF was diluted serially in assay buffer prior to assay. Both the MDK and PTN assays showed good parallelism between the

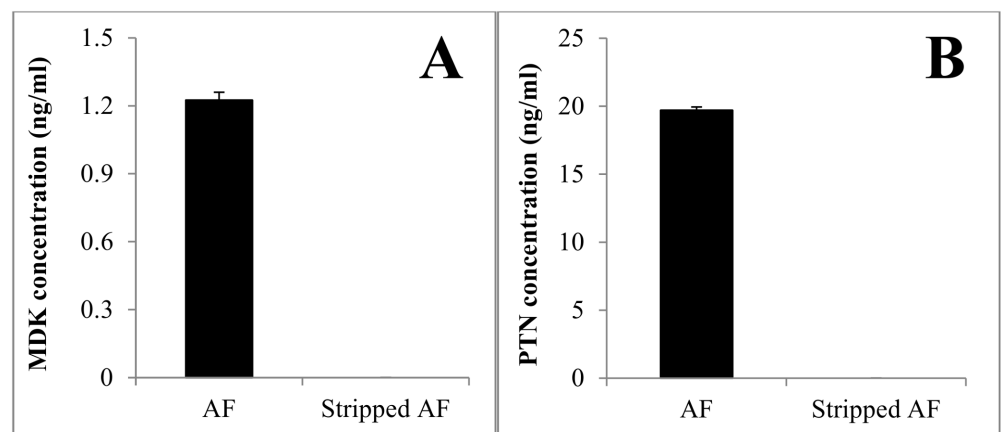


Fig 2. Heparin-stripping of MDK and PTN from amniotic fluid. Assay specificity was assessed by removing both MDK and PTN from AF with heparin-Sepharose beads. ELISA signals for both MDK (Panel A) and PTN (Panel B) were abolished after treatment.

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standard curve and serially diluted AF washout samples (S2A & S2B Fig). A 1:50 dilution of AF was then selected to perform all the MDK and PTN assays.

Binding of MDK and PTN to collection tubes

To determine whether MDK adhered to the glass tube [18–19], blood samples from pregnant women were collected in either glass or polypropylene blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, New Jersey) containing buffered sodium citrate. Plasma MDK concentrations were slightly lower (mean 17%) in the samples collected in glass tubes than in those in polypropylene tubes (S3 Fig). AF samples from the tissue bank had been centrifuged in glass tubes. To determine whether there was a loss of MDK or PTN due to adherence to glass [18–19], freshly collected AF was incubated in either polypropylene or glass tubes at room temperature for 2 hours and assayed. AF MDK concentration was slightly lower (mean 15%) after incubation in glass tubes than in polypropylene tubes, and AF PTN had a higher but still moderate loss (mean 31%) in glass tubes (S4 Fig).

Statistical analysis

All MDK and PTN concentrations were log-transformed. Comparisons of concentrations between pairs of groups to test specific hypotheses (e.g. the effect of chorioamnionitis on growth factor levels at term) were made by *t* test. The association between gestational age and AF growth factor concentrations was examined by a general linear model that included a term for group as depicted in Fig 1B and 1C. Birth weight Z-score was calculated using the Fenton 2013 growth calculator for preterm infants [20–21]. The association between AF growth factor concentration and birth weight was assessed using a general linear model, including terms for gestational age at amniocentesis, gestational age at delivery, and group as covariates. The association between AF MDK and AF PTN was assessed by partial correlation including group as a covariate. Data are presented as mean \pm SEM and were analyzed using SPSS 19 (IBM, NY). A *P* value of ≤ 0.05 was considered statistically significant.

Results

Midkine concentrations in plasma

The average age of the pregnant women at time of plasma sampling was similar to that of the non-pregnant healthy controls [27.6 years (18–40 years) vs. 25.2 years (17–37 years), *P* = 0.18]. Plasma MDK concentrations did not significantly differ between the pregnant women and non-pregnant age-matched controls (0.19 ± 0.01 ng/ml vs. 0.16 ± 0.02 ng/ml, *P* = 0.79). No significant differences in plasma MDK concentrations were found among non-pregnant healthy women, normal mid-term pregnancy, preterm in labor, PPRM, term without labor, and term with labor (Fig 3).

Midkine concentrations in amniotic fluid

In general, MDK concentrations in AF were far higher than in maternal plasma. In healthy term pregnancies in the absence of labor, the average AF MDK concentration was 3.61 ± 1.51 ng/ml while the maternal plasma concentration was 0.18 ± 0.02 ng/ml. MDK concentrations declined with gestational age (*P* < 0.001, Fig 4A). In healthy term pregnancies, AF MDK concentrations were similar between the absence of labor and during labor (3.61 ± 1.51 ng/ml vs. 1.83 ± 0.26 ng/ml, *P* = 0.6, Fig 4B). Interestingly, AF MDK concentrations in term pregnancies complicated by chorioamnionitis were lower than in healthy term pregnancies in the absence of labor (1.12 ± 0.24 ng/ml vs. 3.61 ± 1.51 , *P* = 0.015, Fig 4B). AF MDK concentrations were

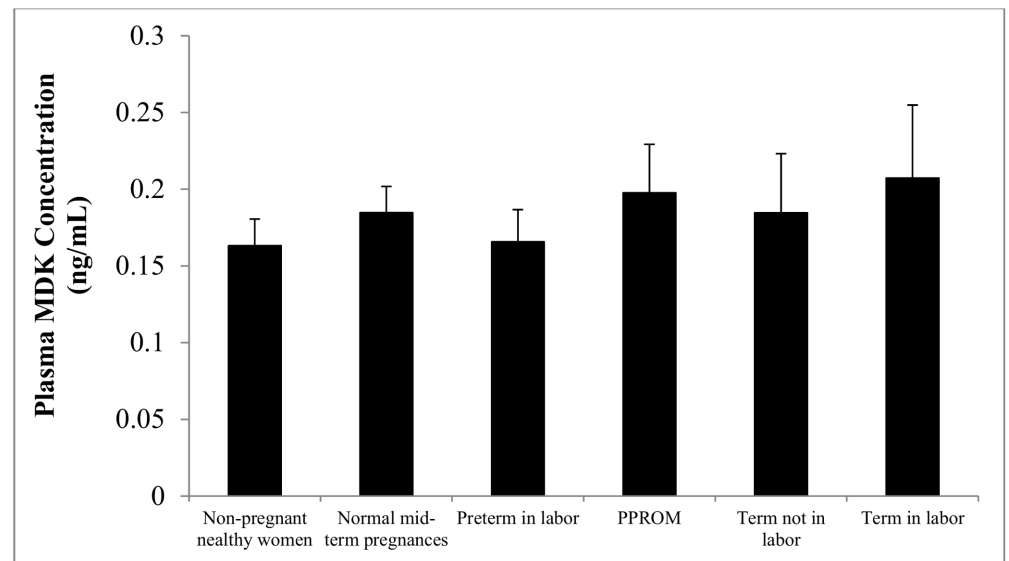


Fig 3. Plasma MDK concentrations during pregnancy and in non-pregnant healthy female controls. Plasma MDK concentrations were similar among non-pregnant healthy women, normal preterm pregnancies in the absence of labor, preterm in labor, PPROM, term not in labor, and term in labor. Data are presented as mean \pm SEM.

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slightly lower in preterm pregnancies during labor than in PPROM (1.61 ± 0.35 ng/ml vs. 1.79 ± 0.28 , $P = 0.046$, Fig 4B).

Pleiotrophin concentrations in amniotic fluid

In healthy term pregnancies without labor, the average PTN concentration in AF was 6.3 ± 1.0 ng/ml. In contrast to AF MDK levels, AF PTN levels did not decline significantly with gestational age ($P = 0.085$, Fig 5A). AF PTN concentrations were similar in healthy term pregnancies in the absence of labor and during labor (6.30 ± 1.00 ng/ml vs. 6.76 ± 1.00 ng/ml, $P = 0.534$). Similar to MDK, AF PTN concentrations in term pregnancies complicated by chorioamnionitis were lower than in healthy term pregnancies in the absence of labor (3.47 ± 0.79 ng/ml vs. 6.30 ± 1.00 ng/ml, $P = 0.01$). AF MDK concentrations were similar between preterm pregnancies during labor and PPROM (3.72 ± 0.77 ng/ml vs. 5.36 ± 1.39 , $P = 0.22$).

Midkine concentrations in amniotic fluid are associated with pleiotrophin concentrations

A positive correlation between AF MDK concentrations and AF PTN concentrations was found for the study cohort ($R = 0.60$, $P < 0.001$, Fig 6).

Discussion

The current study is the first to assess MDK and PTN concentrations in human AF. We found that both of these growth factors could be readily measured in AF at levels greater than those of most cytokines previously assessed in AF [2–5]. The AF concentration of MDK but not PTN declined with gestational age. Both AF MDK and PTN concentrations were lower in pregnancies complicated by chorioamnionitis than in healthy pregnancies. The presence of labor did not affect MDK or PTN levels. We also measured MDK concentrations in plasma and found

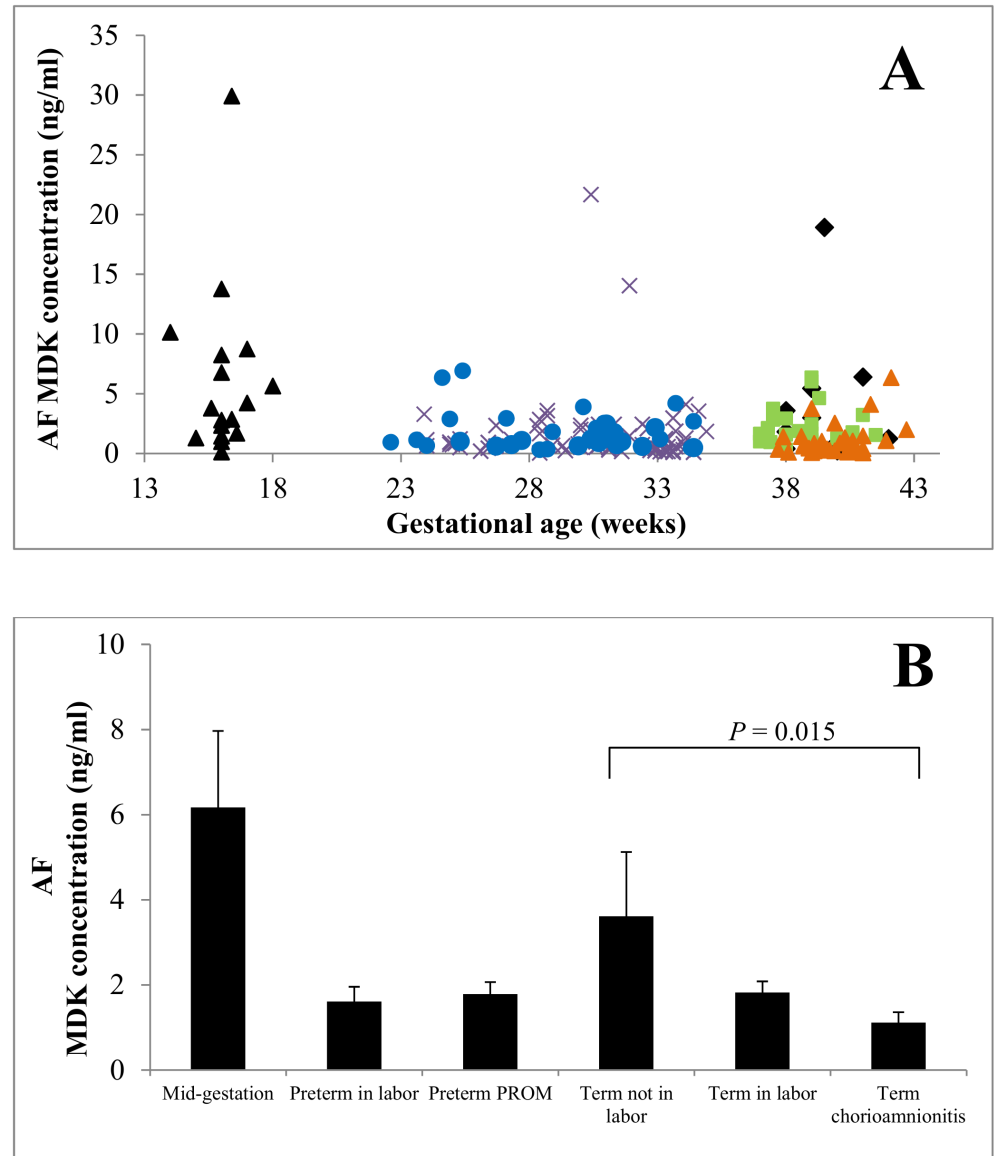


Fig 4. Amniotic fluid MDK concentrations. Panel A: AF MDK concentrations (n = 202) declined with gestational age. Panel B: AF MDK concentrations were not significantly different between healthy term pregnancies in the absence of labor and during labor or between pregnancies complicated by PPROM and premature labor. MDK was lower in term pregnancies complicated by chorioamnionitis than in term pregnancies without infection ($P = 0.015$). Data are presented as mean \pm SEM. Panel A: Black triangle (mid-gestation), x (preterm labor), blue circle (premature preterm rupture of membranes), black diamond (term not in labor), green square (term in labor), orange triangle (term chorioamnionitis).

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that the levels did not differ significantly between pregnant and non-pregnant women. The concentration of MDK was approximately 10-fold higher in AF than in plasma.

In AF, the concentrations of MDK (approximately 2 ng/ml) and PTN (approximately 4.5 ng/ml) were substantially higher than the concentrations of other cytokines previously measured, including IL-6, IL-8, IL-10, IL-11, IL-15, TNF- α , TGF- β , and VEGF. The relative abundance of these two growth factors is consistent with previous observations that the genes encoding both factors are highly expressed in multiple embryonic tissues [11, 13–14, 22–27].

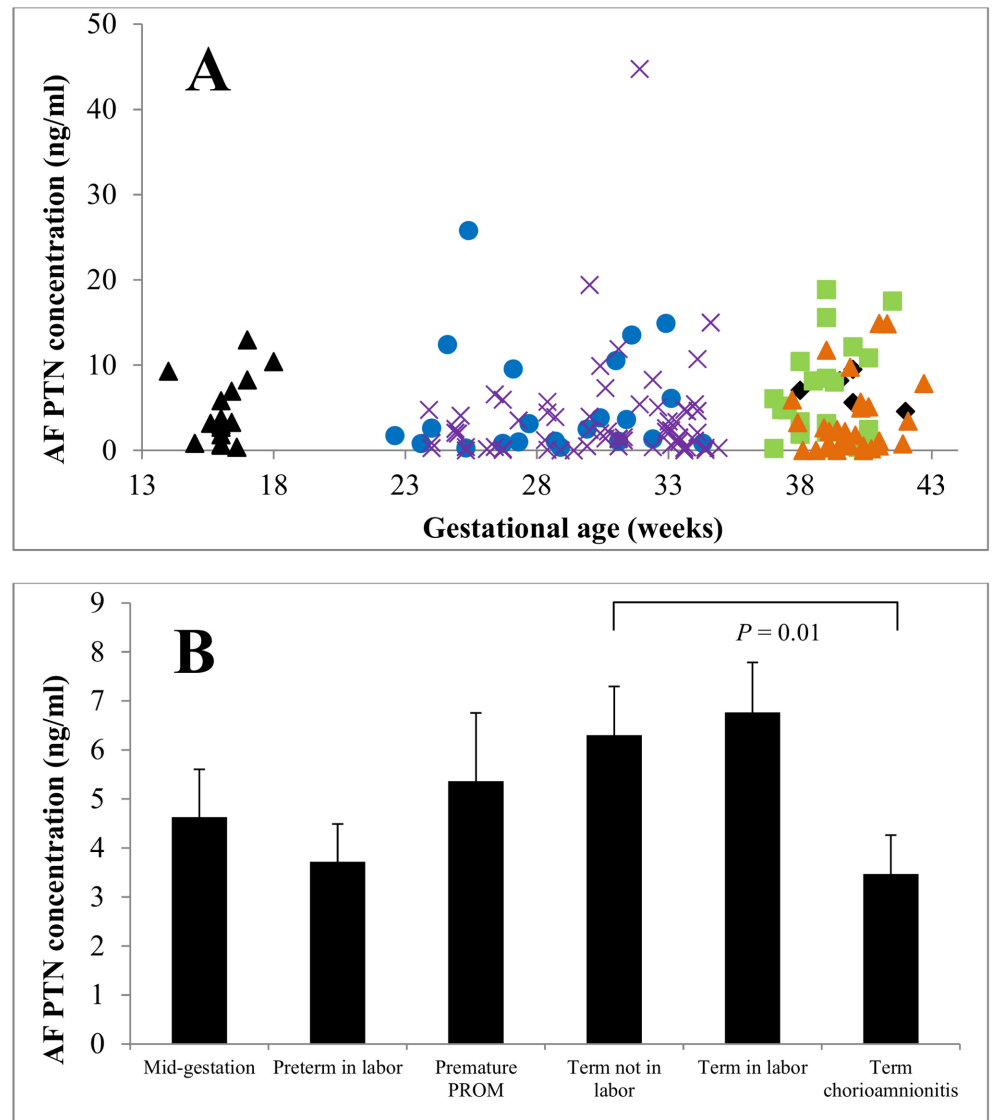


Fig 5. Amniotic fluid PTN concentrations. Panel A: AF PTN concentrations ($n = 170$) did not vary significantly with gestational age (Panel A). Panel B: AF PTN concentrations were similar between the absence and presence of labor in healthy term pregnancies, and also similar between pregnancies complicated by PPRM and by premature labor. MDK was lower in term pregnancies complicated by chorioamnionitis than in term pregnancies without infection ($P = 0.01$). Data are presented as mean \pm SE. Panel A: Black triangle (mid-gestation), x (preterm labor), blue circle (premature preterm rupture of membranes), black diamond (term not in labor), green square (term in labor), orange triangle (term chorioamnionitis).

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In mice, MDK was previously found to be expressed in extraembryonic membranes and present in amniotic fluid at a concentration of approximately 1 $\mu\text{g/ml}$ [28]. Despite the relatively high levels of MDK in AF, its levels in maternal circulation were not elevated compared to its plasma levels in healthy non-pregnant women, suggesting that MDK does not escape from the fetal compartment in substantial quantities. In rodents, expression of both MDK and PTN [13–14] decreases with age in multiple tissues postnatally [10–12, 29–31]. We therefore anticipated that levels of these proteins might decrease with gestational age in human AF. Consistent

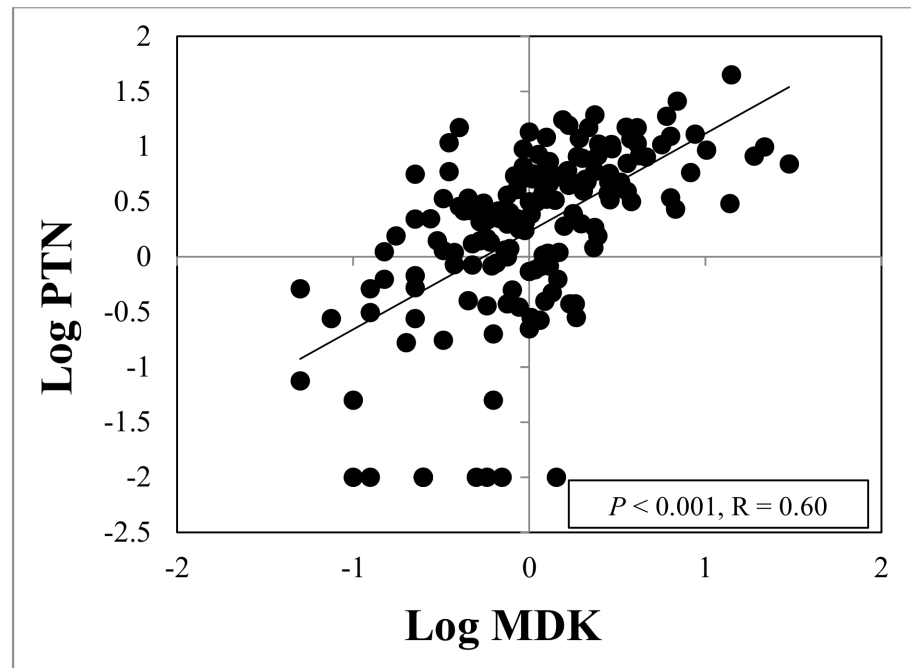


Fig 6. Amniotic fluid midkine and pleiotrophin concentrations correlated with each other. Midkine concentrations were positively correlated with pleiotrophin concentrations in amniotic fluid ($R = 0.6$, $P < 0.001$), using diagnostic category as a covariate.

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with this expectation, MDK AF concentrations declined between mid-gestation and term. However, PTN concentrations did not change significantly with gestational age.

The MDK and PTN found in amniotic fluid may be derived from embryonic and/or extra-embryonic tissues. Both of these heparin-binding growth factors are expressed at high levels in multiple embryonic tissues [12]. In addition, MDK is expressed in the placenta and extra-embryonic membranes of the mouse [26, 28]. PTN expression is low in the trophoblast of most mammals but is highly expressed in the human and chimpanzee, driven by a trophoblast-specific promoter due to insertion of an endogenous retrovirus during primate evolution [27]. Therefore, in humans, amniotic fluid MDK and PTN, which we found in high concentrations, may be derived from the fetus, placenta, and/or amniotic membrane.

The overall and AF-specific functions of MDK and PTN in human development remain unclear. The developmental functions of these growth factors have been investigated in tissue culture and animal models, suggesting roles in the development of the nervous, skeletal, reproductive, and endocrine systems [32–35]. In mice, targeted genetic ablation of both genes results in reduced postnatal growth, infertility, cochlear, auditory dysfunction, and early death [36–37]. PTN may play an important role in the primate placenta. As noted above, there is evidence that insertion of a retrovirus-like element into the primate PTN gene generated an additional promoter with trophoblast specific activity. It has been suggested that the resulting high level of PTN expression may support embryo implantation into the uterus and drive invasion of the trophoblast into the uterine muscle [38–40].

Interestingly, both MDK and PTN concentrations were lower in term samples from pregnancies complicated by chorioamnionitis than in those from healthy pregnancies, suggesting that infection either decreases the expression or accelerates the degradation of these growth factors. This observation also raises the possibility that measuring MDK and PTN in AF might

serve as an adjunctive diagnostic tool to determine the presence of infection. Whether the decreased AF MDK and PTN exert functional effects on the fetus is unknown.

Although this study demonstrates the presence of MDK and PTN in human AF at higher concentrations than other cytokines, the subgroup analysis comparing different gestational ages and different pregnancy complications was limited by the nature of the sample set. For ethical reasons, AF can only be obtained when amniocentesis is clinically indicated. Thus, for example, at 22 to 35 weeks of gestation, we could obtain samples from women with PPRM but not from those with healthy pregnancies. For similar practical reasons, the study used cross-sectional sampling rather than longitudinal sampling from each subject, limiting our ability to analyze the natural history of how the levels of these heparin-binding growth factors are regulated through the course of normal pregnancy. In addition, the samples for this study were obtained from an established tissue bank, which further limited the number, diagnoses, and gestational age of subjects available. For example, our sample population did not include pregnancies complicated by intrauterine growth retardation or overgrowth, a group of considerable interest given the requirement for these two growth factors for normal growth in mice [36]. Furthermore, because the plasma and AF samples were not taken from the same subjects for MDK measurement, the correlation between the two sets of measurement could not be determined. Plasma PTN concentrations were not determined because the assay developed in our lab showed strong interference from matrix effects and consequently poor parallelism. To build upon the findings of this pilot study and to address its limitations, further studies would be of interest, exploring the changes of these growth factor levels with gestational age, their relationships to fetal growth, and their alterations during pregnancy complications that were not addressed in this preliminary study.

Conclusions

In conclusion, we have successfully developed methods to measure both MDK and PTN concentrations in AF and found that both growth factors were present at higher levels than most other cytokines previously measured in AF. For MDK, the levels in AF were also substantially higher than in maternal plasma. MDK but not PTN concentrations declined with gestational age. Both MDK and PTN levels were found to be lower in pregnancies complicated by chorioamnionitis, suggesting that these growth factors might be used clinically as markers for infection. Further investigation is needed to determine the roles of these growth factors in human gestation, particularly whether or not MDK or PTN regulate human growth *in utero* and whether abnormalities involving these growth factors might contribute to intrauterine growth retardation.

Supporting Information

S1 Fig. Parallelism between the standard curve and serially diluted samples of the MDK ELISA. A plasma sample was diluted 2, 10, 50, and 100 fold into assay buffer (TBSTA) and then assayed for MDK. Black bars, measured MDK concentrations; patterned gray bars, measured value multiplied by the dilution factor. Data are presented as mean \pm SEM. (DOCX)

S2 Fig. A & B. Parallelism between the standard curve and serially diluted amniotic fluid washout samples. An amniotic fluid sample was diluted 100, 200, and 400 fold into assay buffer for MDK measurement (Panel A) and 25, 50, and 100 fold for PTN measurement (Panel B). The assays showed good parallelism between the standard curve and serially diluted AF washout samples. Black bars, measured MDK/PTN concentrations; patterned gray bars, measured value multiplied by the dilution factor. Data are presented as mean \pm SEM. (DOCX)

S3 Fig. Comparison of MDK concentrations in plasma collected in polypropylene vs. glass tubes. Plasma in the tissue bank had been collected in glass citrate tubes, centrifuged promptly, and stored at -80°C in polypropylene tubes. To determine whether MDK adhered to the glass tubes (Hando et al., 2008), freshly obtained blood samples ($n = 5$) from pregnant women were collected in either glass or polypropylene blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, New Jersey) containing sodium citrate, incubated for 2 hours at room temperature, and centrifuged. The plasma was then transferred to polypropylene storage tubes and frozen at -80°C until subsequent analysis of MDK. Plasma MDK concentrations (mean \pm SEM of replicates) were slightly higher in polypropylene (black bars) than in glass collection tubes (patterned gray bars). (DOCX)

S4 Fig. A & B. Comparison of MDK and PTN concentrations in AF collected in polypropylene vs. glass tubes. AF in the tissue bank had been centrifuged in glass tubes. To determine whether MDK or PTN adheres to the glass (Hando et al., 2008), freshly collected AF ($n = 5$) was placed in either polypropylene or glass tubes, stored at room temperature for 2 hours, aliquotted into polypropylene tubes, frozen at -80°C , and later assayed for MDK and PTN. AF MDK (Panel A) and PTN (Panel B) concentrations (mean \pm SEM of replicates) were slightly higher in polypropylene (black bars) than in glass collection tubes (patterned gray bars). (DOCX)

Author Contributions

Conceived and designed the experiments: YHJ JB. Performed the experiments: YHJ. Analyzed the data: YHJ YL JB. Contributed reagents/materials/analysis tools: PC GY IP AW RR. Wrote the paper: YHJ YL.

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