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Endoglin in Amniotic Fluid as a Risk Factor for the Subsequent Development of Bronchopulmonary Dysplasia

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Abstract

Objective—Cross-talk between inflammation and angiogenesis pathways has been recently reported. The objective of this study was to: 1) examine whether amniotic fluid (AF) concentrations of soluble endoglin (sEng), a protein with anti-angiogenic properties, changes during pregnancy, parturition or intraamniotic infection and/or inflammation (IAI); 2) determine whether an elevation of sEng in the AF of patients with preterm labor (PTL) and preterm prelabor rupture of membranes (PROM) is associated with adverse neonatal outcomes; and 3) investigate potential sources of sEng in AF.

Study Design—A cross-sectional study was conducted to include patients in the following groups: 1) midtrimester (n=20); 2) PTL with term delivery (n=95); 3) PTL leading to preterm delivery with (n=40) and without IAI (n=46); 4) preterm PROM with (n=37) and without IAI (n=37); 5) term in labor (n=48) and not in labor (n=44). AF concentrations of sEng were determined by ELISA. Chorioamniotic membranes, umbilical cord blood and AF macrophages were examined for the expression of endoglin.

Results—1) Patients with IAI had a higher median AF concentration of sEng than those without IAI (p=0.02 for PTL; and 0.06 for preterm PROM); 2) AF concentrations of sEng in the 3rd and 4th quartiles were associated with IAI (OR 2.5 and 7.9 respectively); 3) an AF sEng concentration ≥779.5 pg/ml was associated with bronchopulmonary dysplasia (BPD) (OR 7.9); 4) endoglin was co-localized with CD14+ macrophages in AF pellets of patients with IAI by immunofluorescence and flow cytometry; and 5) the concentration of sEng in the supernatant was significantly increased after treatment of macrophages with endotoxin or TNF-α.

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Conclusions—sEng participates in the host response against IAI. Activated macrophages may be a source of sEng concentrations in the AF of patients with IAI. An increase of sEng in the AF is associated with BPD and adverse neonatal outcomes.

Keywords

preterm labor; preterm prelabor rupture of membranes; angiogenesis; adverse neonatal outcomes; intraamniotic infection; intraamniotic inflammation

INTRODUCTION

Preterm birth accounts for 75% of perinatal mortality and more than half of the long- term morbidity of survivors.[1-8] Preterm neonates are at increased risk for the development of short-term complications such as respiratory distress syndrome,[9-11] sepsis,[12-16] intraventricular hemorrhage,[17-19] periventricular leukomalacia,[20-22] and necrotizing enterocolitis,[23-25] as well as long-term disabilities, such as cerebral palsy,[26-34] bronchopulmonary dysplasia (BPD),[35-41] and retinopathy of prematurity.[42,43] Intra-amniotic infection and/or inflammation (IAI) has emerged as an important pathologic process for which a firm causal link to preterm parturition is proven.[1,2,44-51]

Infection can occurs in different sites such as the amniotic cavity or placenta. A role for microbial invasion of the amniotic cavity with bacteria in the etiology of complications of pregnancy, such as preterm labor with intact membranes,[52-65] preterm premature rupture of membranes,[66-71] and cervical insufficiency[72-78]is well-established. Human chorioamniotic membranes[79-82] and trophoblasts[83-90]are bestowed with pattern recognition receptors that allow them to recognize a wide range of microorganisms. Infection of the placenta with viruses has been recently demonstrated to elicit an inflammatory response in the fetus even though the organisms have not been recovered from amniotic fluid or fetal tissues[91]. Such infection of the placenta can lead to fetal inflammation and sensitization to bacterial products predisposing to preterm labor.[92] Whatever the precise locations of intrauterine infection are, it can elicit an inflammatory response in the fetus,[5,12,13,19,93-95] chorioamniotic membranes,[81,96-99] amniotic cavity[12,19,47,93,94,100] and placenta.[101-103]

Angiogenesis[104] and inflammation[105] are two distinct processes. Yet, cross-talk between these two processes has been identified in the mechanisms responsible for wound healing,[106] cancer[107] and sepsis.[108,109] Inflammation is a mechanism of host defense to control endogenous or exogenous damage and restore homeostasis in response to infection, bacteria, or tissue injury.[110] This complex process can stimulate angiogenesis, which, in turn, plays an important role in inflammation[107] and its resolution.[110]

Endoglin, also known as CD105, is a co-receptor of transforming growth factor (TGF)-ß and exists in two forms: membrane-bound and soluble.[111-114] Soluble endoglin (sEng) can inhibit TGF-ß activity and has an anti-angiogenic effect on endothelial cells.[114] An elevation of sEng concentration in the maternal circulation[114-120] and in amniotic fluid[121] has been reported in patients with preeclampsia, an obstetrical syndrome proposed to be an anti-angiogenic state.[114,122] Macrophages and neutrophils from patients with a deficiency in endoglin (e.g. hereditary hemorrhagic telangiectasia, a genetic disorder characterized by a

mutation in the endoglin gene and reduced expression of endoglin), have a deficit in phagocytosis and oxidative burst, suggesting a role for endoglin in the regulation of the innate immune response.[123]

One of the most important long-term neonatal complications of preterm birth is BPD and intraamniotic inflammation has been associated with the subsequent development of BPD.[35,40,93,124-127] Recent studies also suggest that BPD is associated with dysregulation of angiogenesis in the pulmonary vasculature.[128-137] Of note, a study reported that the pulmonary microvasculature of ventilated preterm infants, compared to age-matched non-ventilated lungs of control infants, showed significant up-regulation of endoglin mRNA and protein expression.[138] The authors proposed that BPD is associated with a shift in the balance of angiogenic (vascular endothelial growth factor, VEGF, and angiopoietin-1), to alternative regulators such as endoglin, which may contribute to BPD-associated microvascular abnormalities.[138]

The objectives of this study were to: 1) examine whether amniotic fluid concentrations of sEng, a protein with anti-angiogenic activity, change during pregnancy, parturition or IAI; 2) determine if there was a change, whether an elevation of sEng concentration in the amniotic fluid of patients with preterm labor (PTL) and preterm prelabor rupture of membranes (PROM) was associated with adverse neonatal outcomes especially for BPD; and 3) to investigate potential sources of sEng in AF.

MATERIALS AND METHODS

Study design and population

A cross-sectional study was conducted by searching our clinical database and bank of biological specimens, including 367 patients in the following groups: 1) women in the midtrimester of pregnancy who underwent amniocentesis for genetic indications and delivered a normal neonate at term (n=20); 2) patients with an episode of spontaneous PTL and intact membranes who were classified as: a) PTL without IAI who delivered at term (n=95); b) PTL without IAI who delivered preterm (<37 weeks gestation; n=46); and c) PTL with IAI who delivered preterm (n=40); 3) patients with preterm PROM with (n=37) and without IAI (n=37); and 4) normal pregnant women at term with (n=48) and without spontaneous labor (n=44). Patients with multiple pregnancies, preeclampsia, maternal medical disease, fetal death, and fetal congenital or chromosomal abnormalities were excluded.

All patients provided written informed consent upon enrollment. The Institutional Review Boards of both Wayne State University and the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health (NICHD/NIH/DHHS) approved the collection of samples for research purposes. Many of the biological materials of patients who were enrolled in this study have previously been used for studies of inflammation and growth factor concentrations in normal pregnant women and those with pregnancy complications.

Clinical definitions

Spontaneous PTL was defined by the presence of regular uterine contractions (at least four in 20 minutes) associated with cervical changes between 20 and 36 6/7 weeks of gestation and required hospitalization.[139] Preterm PROM was diagnosed by visualization of pooling of amniotic fluid in the vagina in association with positive nitrazine and/or ferning tests or by a positive amniocentesis-dye test before 37 weeks of gestation. Women at term (≥ 37 weeks) not in labor underwent amniocentesis for the assessment of fetal lung maturity prior to cesarean section. Women at term in labor consisted of those admitted for suspected PTL because of uncertain dates and had an amniocentesis for the assessment of fetal lung maturity and microbial invasion of the amniotic cavity. However, those who delivered a neonate ≥2500 g without complications of prematurity were considered likely to represent patients in spontaneous labor at term.[60] IAI was defined as a positive culture for microorganisms and/or an elevated amniotic fluid interleukin (IL)-6 concentration (≥2.6 ng/mL).[94]

Composite neonatal morbidity was defined as the presence of one or more of the following complications: sepsis or suspected sepsis, respiratory distress syndrome, persistent ductus arteriosus, BPD, intraventricular hemorrhage, necrotizing enterocolitis and retinopathy of prematurity. BPD was diagnosed if the neonate required oxygen and ventilatory therapy for >28 days during the first 2 months of life, had typical radiographic changes and/or dysplasia of the bronchopulmonary tree at autopsy.[140,141] The definition of other neonatal complications has been described in previous publications. [11,13,19]

Amniotic fluid collection

Amniotic fluid samples were obtained by transabdominal amniocentesis under ultrasonographic guidance. Samples of amniotic fluid were cultured for the presence of microorganisms, including aerobic and anaerobic bacteria as well as genital *Mycoplasmas*. White blood cell (WBC) count,[142] glucose concentration,[143,144] and Gram stain[145] were also performed. The results of these tests were used for clinical management. Amniotic fluid not required for clinical assessment was centrifuged for 10 min at 4°C, and the supernatant was aliquoted and stored at -70°C until analysis. Among patients with spontaneous PTL and those with preterm PROM who delivered preterm within 72 hours of amniocentesis, the placenta, umbilical cord and chorioamniotic membranes were collected and the presence or absence of histologic chorioamnionitis and/or funisitis was assessed. This interval was chosen to preserve a meaningful temporal relationship between amniotic fluid sEng concentration and placental histopathologic findings.

Determination of sEng concentrations in amniotic fluid and culture supernatants

sEng concentration was measured by a commercial ELISA kit (R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The amniotic fluid concentrations of sEng were determined by interpolation from individual standard curves generated from known concentrations of sEng. The assay was validated for use in amniotic fluid prior to its use in this study. The calculated inter-assay and intra-assay coefficients of variation for sEng in our laboratory were 2.88% and 4.76%, respectively, and the sensitivity was 32 pg/mL.

Flow cytometry

Flow cytometry analysis of amniotic fluid cell pellets was performed to identify if these cells were a source of endoglin. We used a BD^{TM} LSR II flow cytometer (BD Biosciences, San Jose, CA, USA). After incubation with mouse monoclonal anti-human CD14-APC (BD Biosciences, San Jose, CA, USA) and anti-human endoglin-PE (R & D Systems), macrophages were gated as CD14 positive cells. An isotype IgG1 antibody (BD Biosciences, San Jose, CA, USA) matched by concentration was used as the control. The endoglin expression of macrophages (CD14+) in amniotic fluid was analyzed using the FlowJo software (Tree Star, San Carlos, CA, USA).

Immunofluorescence microscopy

Double label immunofluorescence staining on amniotic fluid cell pellets was conducted using a panel of antibodies to endoglin (mouse monoclonal; DakoCytomation Inc., Carpinteria, CA, USA) and CD14 (rabbit polyclonal; Abcam, Cambridge, MA, USA). After fixation with 4% paraformaldehyde and blocking with 5% BSA in PBS, the slides were incubated with a primary antibody to endoglin, followed by incubation with Alexa 594 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) and Alexa 488 donkey anti-rabbit IgG (Invitrogen). The stained slides were mounted in ProLong® Gold antifade reagent with DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride) (Invitrogen), and images were taken using an Olympus BX-60 digital microscope (Olympus Optical Co., Hamburg, Germany). The antibody for the marker of neutrophils was not used because neutrophils can be recognized easily by morphology.

Immunohistochemistry to detect endoglin in the chorioamniotic membranes

Immunohistochemical staining was performed using a mouse monoclonal anti-endoglin antibody (DakoCytomation Inc.) on 5 μm thick paraffin-embedded sections of the chorioamniotic membranes with an automatic immunostainer (Ventana Benchmark; Ventana Medical Systems, Inc., Tucson, AZ, USA).

Immunoblotting and densitometry to examine the expression of endoglin in chorioamniotic membranes

Immunoblotting was performed using chorioamniotic membranes obtained from PTL with (n=8) and without (n=8) histologic chorioamnionitis. Total protein was obtained from liquid nitrogen-pulverized fetal membranes using a RIPA (radio-immunoprecipitation assay) lysis buffer (Sigma, St Louis, MO, USA) containing a proteinase inhibitor cocktail (Roche, Indianapolis, IN, USA). Thirty micrograms of protein were electrophoresed in a 12% SDS-PAGE gel (Bio-Rad, Hercules, CA, USA) and electro-blotted to polyvinylidene difluoride membranes (Hybond™-P; GE Healthcare Life Sciences, Piscataway, NJ, USA). After blocking with 5% blotting grade blocker non-fat dry milk (Bio-Rad), the membranes were probed with a goat polyclonal anti-endoglin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or a rabbit polyclonal anti-HPRT antibody (Santa Cruz Biotechnology Inc.). The chemiluminescent signals were detected using a ChemiGlow West kit (Alpha Innotech, San Leandro, CA, USA). Densitometric analyses were carried out using AlphaEase®FC software version 4.1.0 of the FluorChemTM SP densitometer (Alpha Innotech).

Expression of endoglin by a macrophage cell line

U937 cells (ATCC, Manassas, VA, USA), a human promonocytic cell line, were used as a model for macrophage-like cells. We cultured at 37°C in a 5% CO_2 atmosphere in RPMI-1640 medium supplemented with 2.5g/L glucose, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1 mM sodium pyruvate, 10% fetal bovine serum and 100 μg/mL of penicillin-streptomycin. U937 cells were incubated with 10 ng/mL of phorbol myristic acid (PMA) for 48 hours to induce differentiation into adherent macrophage-like cells. Following the PMA treatment, the medium was replaced with a fresh one, and the differentiated cells were incubated for an additional 24 hours prior to the conduction of the studies.

To determine whether microbial products and pro-inflammatory cytokines can induce the release of sEng by macrophage-like cells, we incubated U937-derived macrophages with 1 μg/mL of lipopoylsaccharide (LPS; Sigma) or 0.1 μg/mL recombinant human tumor necrosis factor (TNF)-α (R & D Systems). Endotoxin and TNF-α have been previously found in the amniotic fluid of women in preterm labor.[47,146,147] The supernatants and the cells were collected after 24 hours of incubation. sEng was determined by an ELISA previously described above.

Real-time quantitative reverse transcription-polymerase chain reaction

To examine the effect of sEng on the production of cytokines by macrophages, U937-derived macrophages were treated in the presence or absence of recombinant human sEng (R & D Systems; at final concentrations of 0.1, 1 and 2 μg/mL). The supernatants and cells were collected after 24 hours of incubation. Total RNA from the cells was isolated using Trizol (Invitrogen). Reverse transcription of the DNase-treated total RNA was performed using a SuperScript III reverse transcriptase (Invitrogen) and oligo (dT) primers according to the manufacturer's instructions. qRT-PCR analysis using TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) was performed on U937-derived macrophages to determine the mRNA expression of IL-1ß (Hs00174097_m1) and TNF-α (Hs00174128_m1) with RPLPO (4326314E) as an internal control. The concentrations of IL-1ß and TNF-α in culture supernatants were measured by commercially available ELISA (R & D Systems).

Statistical analysis

The distribution of the data was tested using the Shapiro-Wilk test. Non-parametric statistics were used for analyses. Proportions were compared using Chi-square or Fisher's exact tests. A Kruskal-Wallis with *post-hoc* Mann-Whitney *U* test was utilized for comparisons among and between continuous variables. Correlations between continuous variables were assessed by the Spearman's rank correlation test. Associations between the amniotic fluid concentration of sEng and the presence of IAI, or neonatal outcomes (BPD and composite neonatal morbidity) were determined by logistic regression (backward-stepwise) adjustment for potential confounders. A p-value of <0.05 was considered statistically significant. The statistical analyses were performed using SPSS version 12.0 (SPSS Inc, Chicago, IL, USA).

RESULTS

Demographic and clinical characteristics of the study population

Table I, II, and III present the demographic and clinical characteristics of patients included in this study. There was no significant difference in the median gestational age at amniocentesis among subgroups of patients with spontaneous PTL (Table II) or preterm PROM (Table III).

Intraamniotic infection/inflammation is associated with a change in the amniotic fluid concentrations of sEng

Patients with spontaneous PTL with IAI had a significantly higher median amniotic fluid concentration of sEng than those who delivered preterm without IAI (p=0.02) and those who delivered at term (p=0.003; Figure 1A). Similarly, among patients with preterm PROM, the median amniotic fluid concentration of sEng was higher in patients with IAI than in those without IAI, but the difference did not reach statistical significance (p=0.06; Figure 1B). The amniotic fluid concentration of sEng was positively correlated with the amniotic fluid concentration of IL-6 and the amniotic fluid WBC count (Spearman's rho 0.4 and 0.3; respectively, each p<0.001).

Logistic regression analysis indicated that amniotic fluid concentrations of sEng in the 3rd (336-545 pg/mL) and 4th quartiles (>545 pg/mL) were associated with IAI after adjusting for gestational age at amniocentesis, the presence of preterm PROM, the presence of SGA, smoking, nulliparity, maternal age and sample storage time [odds ratio (OR) 2.5; 95% confidence interval (CI) 1.1-5.5 and OR 7.9; 95% CI 3.7-16.9, respectively].

Among patients with spontaneous PTL and those with preterm PROM who delivered preterm within 72 hours of amniocentesis, placental pathology was available in 73.5% (61/83) of cases. Patients with histologic chorioamnionitis had a higher median amniotic fluid concentration of sEng than those without histologic chorioamnionitis (p<0.001; Figure 1C).

Amniotic fluid concentrations of sEng in normal pregnancies

Women in the midtrimester of pregnancy had a significantly higher median amniotic fluid concentration of sEng than those at term who were not in labor (p<0.001; Figure 1D). There was no significant difference in the median amniotic fluid concentration of sEng between women with spontaneous labor at term and those not in labor at term (p=0.1; Figure 1D).

Neonatal outcomes and amniotic fluid concentrations of sEng in patients with PTL and preterm PROM

The prevalence of BPD and composite neonatal morbidity in patients with PTL and preterm PROM was 5.5% (14/255) and 39.6% (101/255), respectively. Receiver-operating characteristic (ROC) curves of the amniotic fluid concentrations of sEng for the identification of BPD and composite neonatal morbidity are displayed in Figures 2A and 2B. Amniotic fluid sEng concentrations ≥ 779.5 pg/mL in patients with PTL and preterm PROM were associated with BPD (OR 7.9; 95% CI 2.4-26.0, p<0.001) after adjustment for

gestational age at delivery, antenatal steroid administration, the presence of preterm PROM, the presence of SGA, smoking, nulliparity, maternal age and sample storage time. Similarly, amniotic fluid sEng concentrations ≥ 346.5 pg/mL were associated with composite neonatal morbidity (OR 2.2; 95% CI 1.1-4.3, p=0.02).

Amniotic fluid macrophages as a potential source of sEng in the amniotic fluid of patients with intraamniotic infection/inflammation

The potentially ubiquitous nature of endoglin expression and previous observation of endoglin expression in macrophages[148] led us to examine whether amniotic fluid leukocytes express endoglin. Immunofluorescence staining was performed on amniotic fluid cell pellets of patients with IAI, which demonstrated that endoglin was expressed only in CD14+ macrophages (Figure 3), but not in epithelial cells or neutrophils (Figure 4). Furthermore, endoglin was not expressed on the neutrophils in the flow cytometry experiment (Figure 5).

Using flow cytometry, CD14+ macrophages were identified in the amniotic fluid of patients with IAI (Figure 6). In accordance with the results of immunofluorescence staining, CD14+ macrophages expressed endoglin if they were obtained from patients with IAI (Figure 6C). In contrast, CD14+ macrophages were rarely detected in the amniotic fluid of patients without IAI (Figure 6A), and endoglin expression was not detected by flow cytometry.

To confirm that macrophages can release sEng, U937-derived macrophages were treated with either LPS or recombinant TNF-α. The concentration of sEng in the supernatant was significantly increased after treatment with LPS or TNF-α (LPS vs. control: p=0.002; TNF-α vs. control: p=0.03; Figure 7A).

The expression of endoglin and sEng in chorioamniotic membranes and umbilical cord blood

Immunohistochemical staining of the chorioamniotic membranes demonstrated that endoglin was expressed in chorionic trophoblasts and in the endothelial cells of decidual vessels, but not in amnion cells (Figure 7B). A comparison of chorioamniotic membranes obtained from the placentas of patients with PTL with (n=8), and without (n=8) histologic chorioamnionitis showed no significant difference in endoglin or sEng expression on densitometric analysis (endoglin: p=0.14 and sEng: p=0.25; Figure 7C). Moreover, there was no significant difference in the median plasma concentrations of sEng in umbilical cord blood from patients who delivered preterm with (n=40) and without (n=40) histologic chorioamnionitis and funisitis, matched (within 2 weeks) for gestational age at delivery (p=0.6; Figure 7D).

The effect of endoglin on IL-1β and TNF-α production by macrophages

U937-derived macrophages were treated in the presence or absence of recombinant endoglin (0.1, 1 and 2 μg/mL). The mean mRNA expression of IL-1β was significantly increased after treatment with recombinant endoglin at concentrations of 1 and 2 μg/mL ($p<0.05$ for each; Figure 8A). The mean mRNA expression of TNF-α was also increased after adding recombinant endoglin, but the difference did not reach statistical significance (Figure 8B). Treatment of U937-derived macrophages with recombinant endoglin increased the mean

concentration of IL-1β and TNF-α in supernatant in a dose-dependent manner ($p<0.05$ for each; Figures 8C and 8D).

DISCUSSION

Principal findings of the study

1) IAI was associated with an increase in the amniotic fluid concentrations of sEng; 2) an increase in the amniotic fluid concentrations of sEng was associated with histologic chorioamnionitis, BPD and composite neonatal morbidity in patients with PTL and preterm PROM; 3) endoglin was expressed in chorionic trophoblasts and on amniotic fluid macrophages; 4) treatment of macrophages with endotoxin or TNF-α increases sEng concentrations in the culture supernatant; 5) sEng increased mRNA and protein IL-1β and TNF-α production by macrophages; and 6) amniotic fluid concentrations of sEng decreased as a function of gestational age and did not change with spontaneous labor at term.

Intraamniotic infection/inflammation is associated with an increase in the amniotic fluid concentrations of sEng

This is the first study to demonstrate that the amniotic fluid concentration of sEng is higher in patients with IAI than in those without IAI. This finding is in contrast with that of soluble vascular endothelial growth factor receptor (sVEGFR)-1, another important anti-angiogenic factor, which does not increase in the AF of patients with IAI.[149] Furthermore, the amniotic fluid concentration of sEng was positively correlated with indirect markers of IAI, including IL-6 concentration and WBC count in amniotic fluid. These findings are consistent with the results of previous observations, in which endoglin expression was up-regulated in inflammatory conditions. For example: 1) endoglin is strongly expressed in inflamed skin, and this is accompanied by infiltration of macrophage and T cells;[150] and 2) endoglin is highly expressed in the vascular endothelium of cirrhotic livers, and inflamed bowel and lung.[150]

Endoglin and the innate immune response: bridging the gap between inflammation and angiogenesis

An emerging theme in biology is that inflammation is linked to angiogenesis.[107,151] Indeed, inflammation can stimulate angiogenesis: nutrients and oxygen are supplied from the formation of new vessels to allow the transport of inflammatory cells during inflammatory processes. Of note, changes in angiogenic and anti-angiogenic factors expression have been observed in patients with sepsis or those with localized infection. For example, circulating concentrations of angiogenic factors such as VEGF and placental growth factor (PlGF) were increased in patients with sepsis.[109,152] The serum concentration of soluble VEGF receptor-1, which has an anti-angiogenic effect on endothelial cells, correlated with disease severity in sepsis.[153] Moreover, we have demonstrated that IAI is also associated with elevated amniotic fluid concentrations of angiopoietin-2, one of the major regulators of angiogenesis,[154] and acute pyelonephritis during pregnancy changes the balance of angiogenic and anti-angiogenic factors in maternal plasma.[155]

Endoglin has been implicated in the pathophysiology of anti-angiogenic states of pregnancy, such as preeclampsia,[114-116,119,156] SGA,[116] fetal death,[157] preterm labor,[158] twin to twin transfusion syndrome,[159] mirror syndrome,[160] and other conditions such as tumors[161] and hereditary hemorrhagic telangiectasia.[162] The latter is a rare genetic disease, characterized by mutations in the endoglin gene, resulting in low endoglin expression and abnormalities of the blood vessels leading to epistaxis, telangiectasia, and visceral arteriovenous malformations.[163] Although the disease is characterized mainly by abnormal vascular structures, severe infections (such as cerebral abscesses, extra-cerebral infections, hepatic, renal and splenic abscesses) have been reported.[163] Indeed, polymorphonuclear leukocytes and monocytes from patients with hereditary hemorrhagic telangiectasia had reduced phagocytosis and oxidative burst activity.[123] Thus, it is possible that the decreased production of endoglin may be associated with impairment in the innate immune response.

sEng and Bronchopulmonary dysplasia

The findings of this study demonstrate, for the first time, that high concentrations of sEng in amniotic fluid are associated with subsequent development of BPD in neonates. sEng has an anti-angiogenic effect by inhibition of TGF-ß activity.[114] There is evidence that BPD is associated with dysregulation of angiogenesis in the pulmonary vasculature. Evidence in support of this includes: (1) angiogenesis in the lung promotes active alveolarization;[134,137] (2) VEGF and TGF-β signaling are disrupted in BPD;[128,131,164-166] (3) in animal experiments, recombinant VEGF treatment and adenovirus-mediated VEGF gene therapy prevent alveolar injury in BPD;[132,135,136] (4) neonatal treatment with VEGF inhibitor (SU5416) impairs lung growth and decreases nitric oxide production in neonatal rat lungs:[133] in contrast, treatment with inhaled nitric oxide restores lung structure in eNOS-deficient mice;[129] (5) similarly, intraamniotic administration of sVEGFR-1, a potent anti-angiogenic factor, to mice in preterm gestations can decrease the alveolar number, reduce pulmonary vessel density, suppress activation of lung VEGF receptor-2 and increased apoptosis in endothelial and mesenchymal cells in the newborn lung, suggesting that an elevation of anti-angiogenic factors in the amniotic fluid may result in impaired alveolarization and pulmonary vascular growth and contribute to the increased risk of BPD;[167] (6) a high concentration of the anti-angiogenic factor endostatin in umbilical cord blood is associated with the development of BPD in very low birth weight infants;[130] and (7) a significant increase in the expression of endoglin transcript and protein was observed more frequently in the lungs of ventilated preterm infants than in age-matched non-ventilated control lungs.[138]

What are the potential sources of elevated amniotic fluid concentration of sEng in patients with intraamniotic infection/inflammation?

Our findings that endoglin was expressed on amniotic fluid macrophages are consistent with previous studies which identified endoglin expression on the surface of interstitial macrophages from the red pulp of the spleen and on differentiated monocytes.[148,168] Of note, endoglin is weakly expressed or absent on freshly isolated monocytes, while it is readily detectable on tissue and *in vitro*-cultured macrophages.[148,168] Indeed, endoglin expression of maternal monocytes was very weak compared to that of amniotic fluid macrophages obtained from the same patients using flow cytometry (data not shown).

Collectively, the results of previous studies coupled with our findings indicate that endoglin is expressed on CD14+ cells in amniotic fluid, and that these cells are generally present only in patients with IAI (but not in those without IAI).

Other potential sources of sEng in amniotic fluid examined in this study are chorioamniotic membranes and umbilical cord blood. Endoglin expression was observed on chorionic trophoblasts and on the endothelial cells of decidual vessels, but not on amnion epithelial cells, by immunohistochemical staining. However, there was no significant difference (as determined by immunoblotting) in the mean endoglin expression in the chorioamniotic membranes from placentas with and without histologic chorioamnionitis. In addition, there was no significant difference in the median plasma concentration of sEng in umbilical cord blood from preterm neonates with and without histologic chorioamnionitis and funisitis. Therefore, these findings suggest that amniotic fluid macrophages, but not chorioamniotic membranes or umbilical cord blood, may be a source of elevated sEng concentrations in the amniotic fluid of patients with IAI.

Macrophages can release sEng in response to microbial products or pro-inflammatory cytokines

The mechanisms involved in the generation of the soluble form of endoglin are incompletely understood. However, sEng is thought to be released by proteolytic cleavage of the membrane-bound endoglin.[114] The concentrations of sEng in the supernatant of U937-derived macrophages were increased after stimulation with LPS or TNF-α. The findings of this study are novel, and provide evidence that macrophages can release sEng in response to a microbial product or pro-inflammatory cytokines.

sEng increases IL-1β and TNF-α production by macrophages

There is a paucity of information about the role of sEng in the inflammatory response, although several studies have shown its anti-angiogenic effects in the pathophysiology of preeclampsia.[114,115] Our findings demonstrate a novel observation, that sEng increases the production of IL-1β and TNF-α by macrophages. This suggests that the increased sEng concentrations observed in inflamed amniotic fluid may participate in the inflammatory response by stimulating the production of pro-inflammatory cytokines by macrophages. Although the precise mechanisms of cytokine production by sEng were not examined in this study, one plausible mechanism involves TGF-β.

TGF-β has anti-inflammatory properties. Previous studies have supported an important role for TGF-β in the suppression of macrophage cytokine production.[169,170] Werner et al.[171] have demonstrated that deactivation of macrophages by TGF-β1 is mediated via Smad3 signaling through inhibiting the activities of pro-inflammatory transcriptional factors such as NFκB and activating protein-1. sEng can inhibit TGF-β activity, which attenuates eNOS activation and, therefore, has an anti-angiogenic effect on endothelial cells.[114] Further studies are required to elucidate the interactions of endoglin and other pro-inflammatory or anti-inflammatory cytokines.

sEng concentration in amniotic fluid decreases with advancing gestational age in normal human pregnancy

The changes of sEng concentration in amniotic fluid with advancing gestational age in normal pregnancy have not been previously reported. Vascular remodeling and angiogenesis are required for fetal development and normal pregnancy outcome.[172,173] The finding that the amniotic fluid concentration of sEng in the midtrimester was higher than in term gestation suggests a role for sEng during early pregnancy and fetal development. Similar changes have been observed for PlGF[174] and angiopoietin-2.[154] As normal pregnancy approaches term, angiogenesis in the uterus may decrease.

Conclusions

sEng concentrations in amniotic fluid are higher in patients with IAI than in those without IAI. A potential source of sEng in IAI is amniotic fluid macrophages, which release sEng in response to microbial products. sEng, in turn, can stimulate IL-1β and TNF-α production by macrophages. An elevation of sEng in the amniotic fluid is associated with the subsequent development of BPD in neonates. Given these observational and experimental findings, we propose that sEng participates in the intraamniotic inflammatory response in preterm parturition and may contribute to the development of BPD in infants who delivered preterm.

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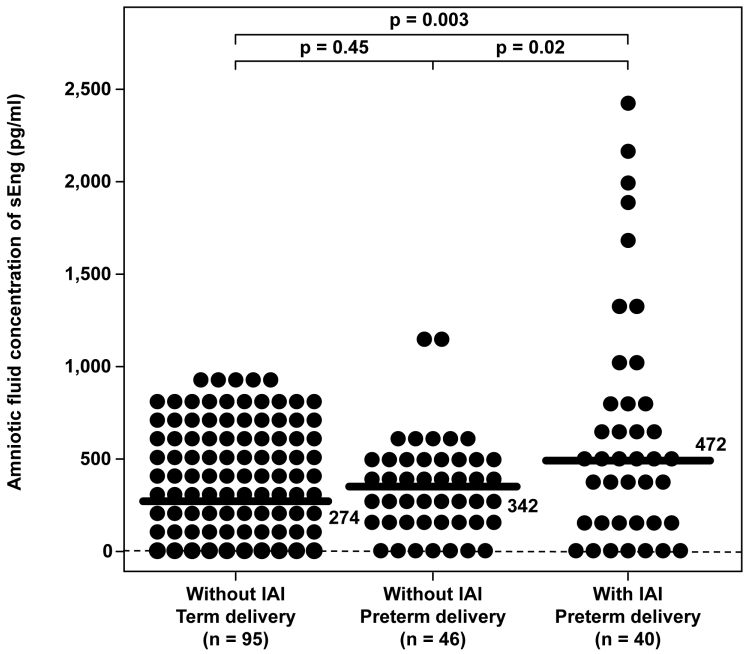
A



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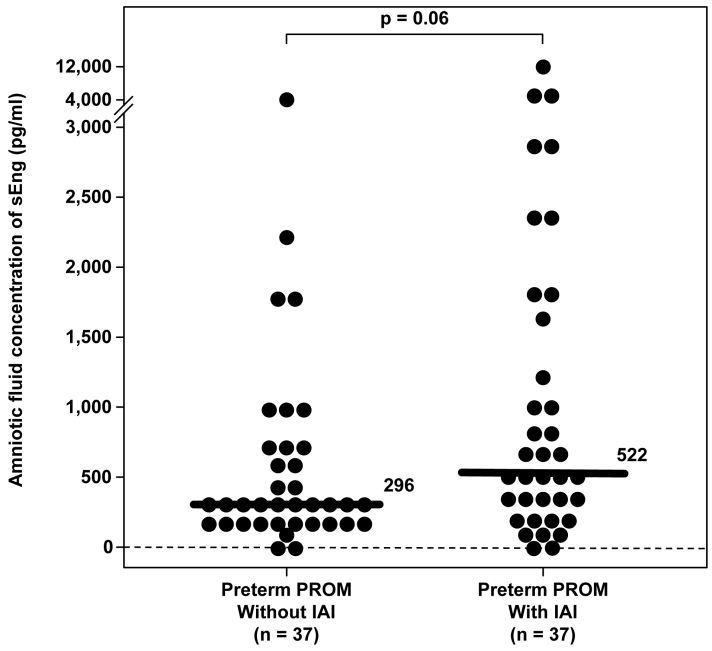

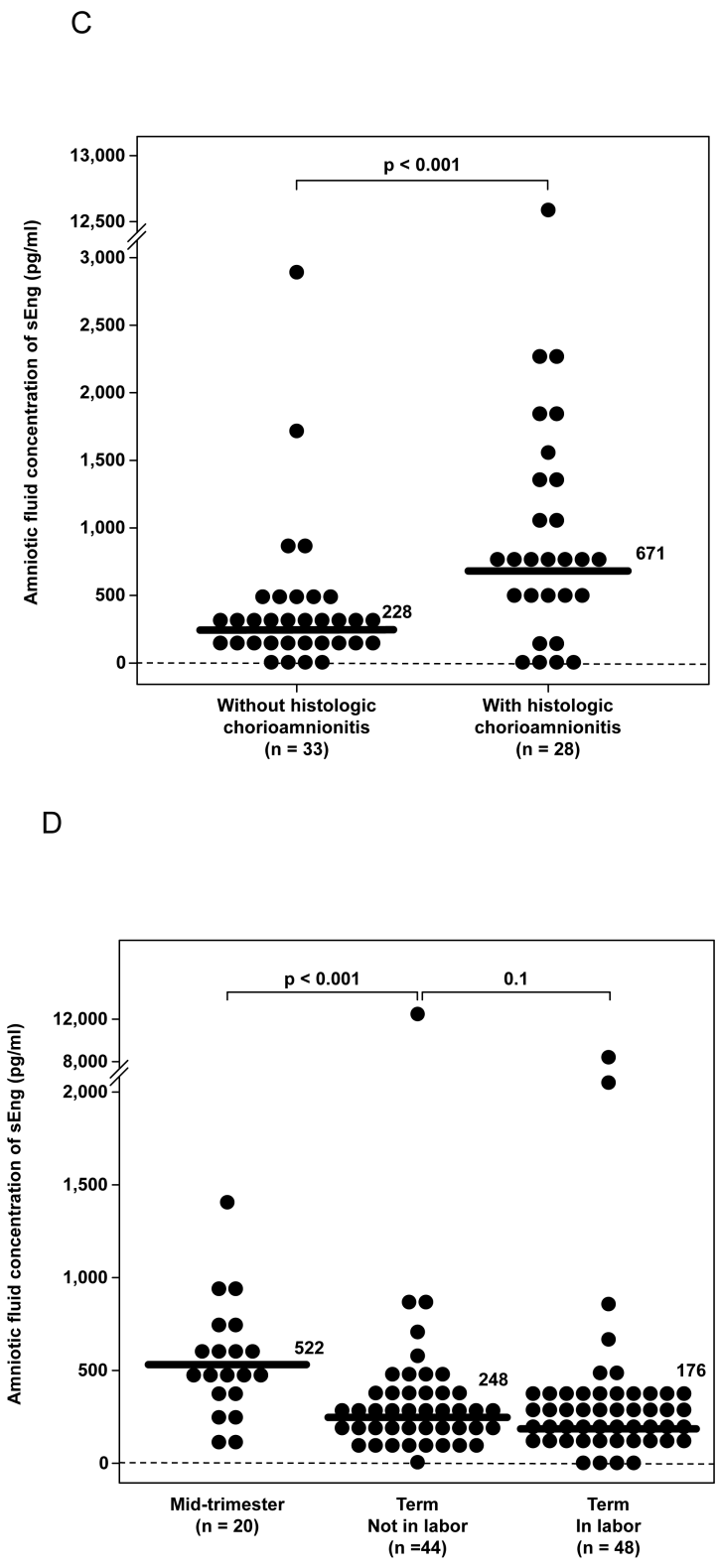

Figure 1. Amniotic fluid concentrations of soluble endoglin (sEng)

(A) Patients with preterm labor (PTL) with intra-amniotic infection/inflammation (IAI) had a significantly higher median amniotic fluid concentration of sEng than those who delivered preterm without IAI [PTL with IAI: 472 pg/mL, interquartile range (IQR) 213-795 pg/mL vs. PTL without IAI: 342 pg/mL, IQR 166-468 pg/mL; p=0.02] and those who delivered at term (PTL delivered at term: 274 pg/mL, IQR 157-405 pg/mL; P=0.003). Among patients with PTL without IAI, there was no significant difference in the median AF concentration of sEng between those who delivered preterm and those who delivered at term (p=0.45). (B) The median amniotic fluid concentration of sEng was higher in patients with IAI than in those without IAI, but the difference did not reach statistical significance (522 pg/mL, IQR 239-1687 vs. 296 pg/mL, IQR 211-626, respectively; p=0.06). (C) Patients with histologic chorioamnionitis had higher median amniotic fluid concentration of sEng than those without histologic chorioamnionitis (671 pg/mL, IQR 419-1334 vs. 228 pg/mL, IQR 128-435, respectively; p<0.001). (D) The median amniotic fluid concentration of sEng was significantly higher in pregnancies in the midtrimester than in those with at term not in labor (522 pg/mL, IQR 351-685 vs. 248 pg/mL, IQR 144-357, respectively; p<0.001). In contrast, no significant differences were observed in the median amniotic fluid sEng concentration between women with spontaneous labor at term and those at term not in labor (176 pg/mL, IQR 124-322 vs. 248 pg/mL, IQR 144-357, respectively; p=0.1).

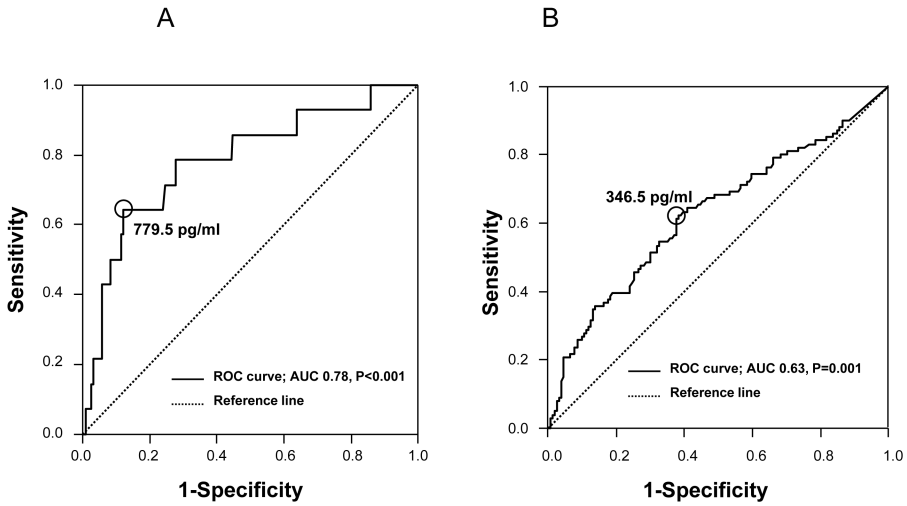

Figure 2.

Receiver-operating characteristic (ROC) curves of amniotic fluid concentrations of soluble endoglin for the identification of bronchopulmonary dysplasia (a; AUC, 0.78; $P < 0.001$) and composite neonatal morbidity (b; AUC, 0.63; $P = 0.001$) in patients with preterm labor and preterm prelabor rupture of membranes. AUC = area under the curve.

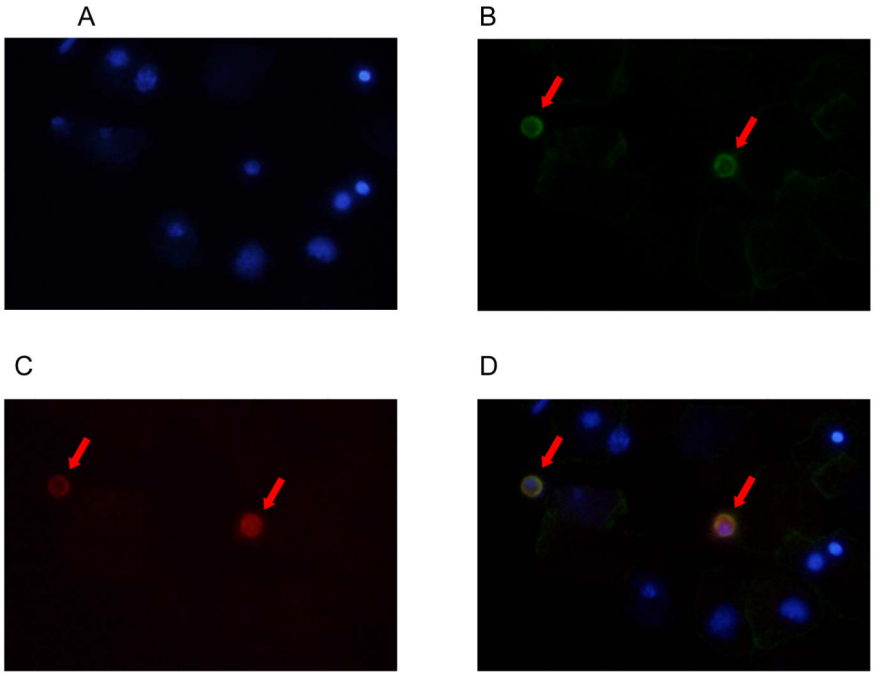

Figure 3. Double-label immunofluorescence staining of an amniotic fluid cell pellet from a patient with intra-amniotic infection/inflammation using antibodies to endoglin and CD14

(A) DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride) staining of nuclei (blue). (B) CD14 expression on macrophages (green; Alexa Fluor 488). (C) Endoglin expression on macrophages (red; Alexa Fluor 594). (D) The merged image showing that macrophages expressed both CD14 (green) and endoglin (red).

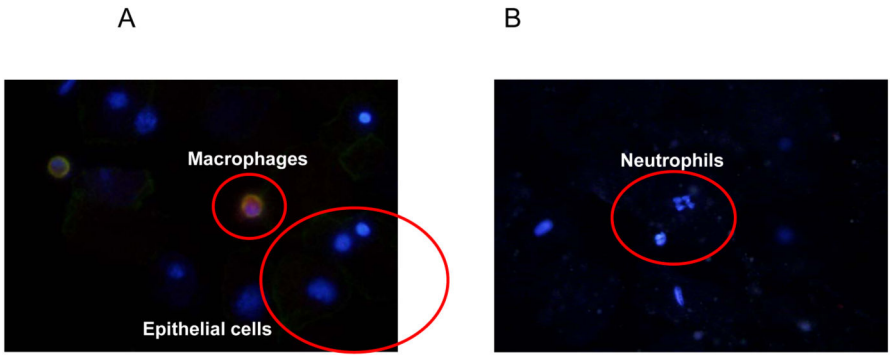

Figure 4. Double-label immunofluorescence staining of an amniotic fluid cell pellet from a patient with intra-amniotic infection/inflammation using antibodies to endoglin and CD14

Endoglin was expressed on CD14+ macrophages (A), but not on epithelial cells (A) or neutrophils (B).

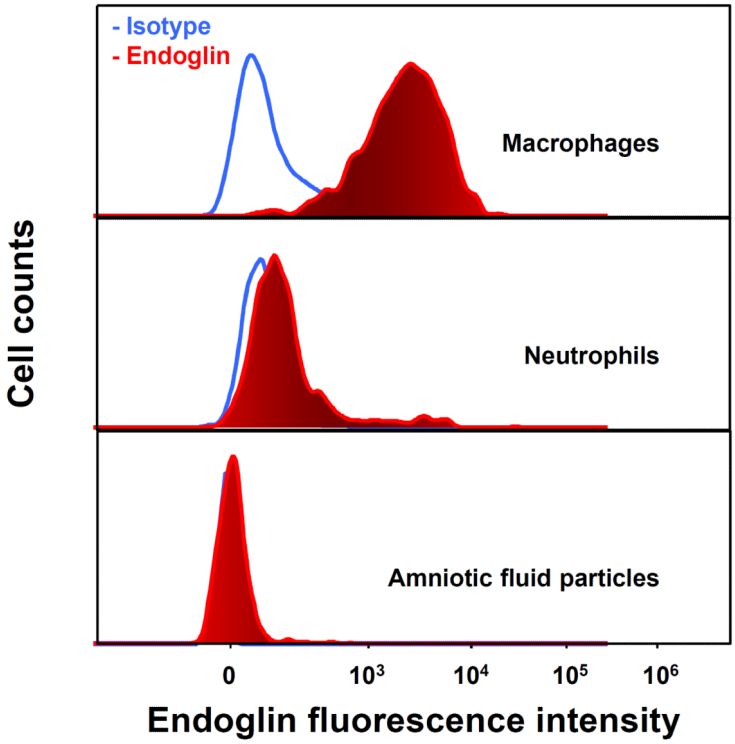

Figure 5. Flow cytometry analysis of endoglin expression on different amniotic fluid (AF) cell subsets from an AF cell pellet

Endoglin was expressed on macrophages (CD14+), but not on neutrophils or AF particles.

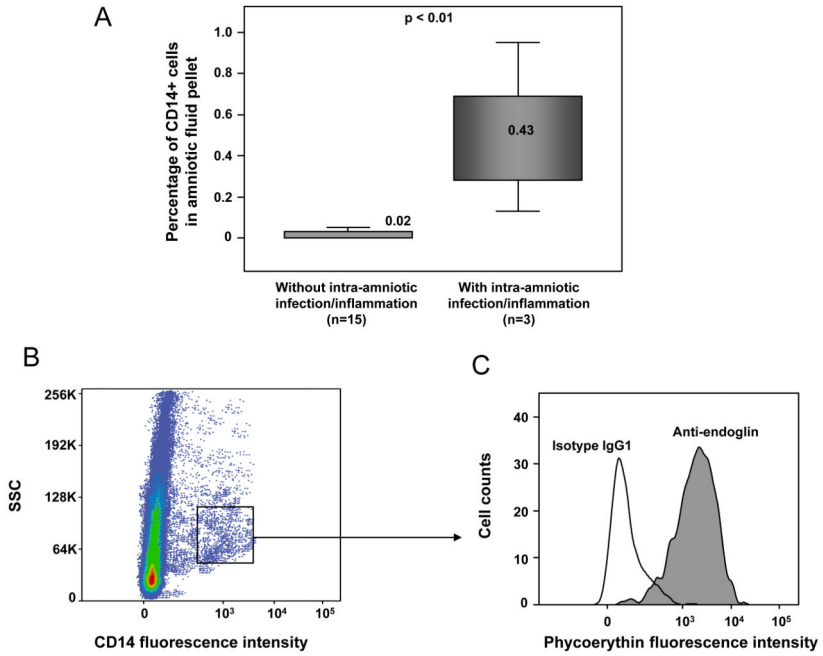

Figure 6. Flow cytometry analysis of endoglin and macrophages (CD14+) in amniotic fluid cell pellets of patients with and without intra-amniotic infection/inflammation (IAI)

The median percentage of CD14+ cells was significantly higher in patients with IAI than in those without IAI (median 0.43 range 0.13-0.95 vs. median 0.02 range 0-0.11; $p<0.01$; see panel A). A subset of macrophages or CD 14+ cells expressed endoglin (see panel B and C).

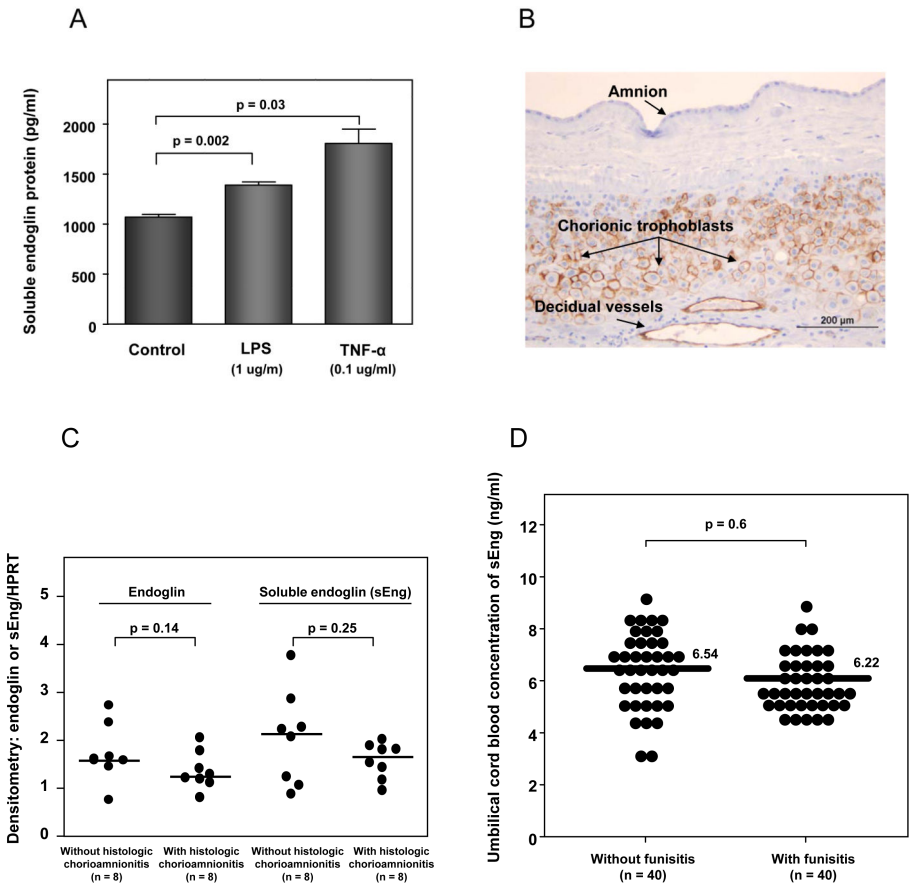

Figure 7. Potential sources of an increased soluble endoglin (sEng) in the amniotic fluid of patients with intra-amniotic infection/inflammation

(A) amniotic fluid macrophages: the mean concentration of sEng in supernatant of U937-derived macrophages was significantly higher than in the control after treatment with LPS (p=0.002) or TNF-α (p=0.03). Data represents mean ± SEM. All the experiments were done in triplicate. The p-values were calculated by Student's t-tests. **(B) chorioamniotic membranes**: immunohistochemical staining of chorioamniotic membranes demonstrated that endoglin was expressed in chorionic trophoblasts and endothelial cells of decidual vessels, but not in amnion cells. **(C) chorioamniotic membranes**: densitometric analysis of chorioamniotic membranes for the expression of endoglin and sEng over HPRT (hypoxanthine-guanine phosphoribosyltransferase) between patients with preterm labor (PTL) with (n=8) and without (n=8) histologic chorioamnionitis. There was no significant difference in the median endoglin density ratio and sEng density ratio between patients with and without histologic chorioamnionitis [endoglin density ratio: median 1.24, interquartile range (IQR) 1.14–1.58 vs. median 1.58, IQR 1.49-2.0, p=0.14; sEng density ratio: median 1.66, IQR 1.29–1.83 vs. median 2.13, IQR 1.14–2.55, p=0.25]. **(D) umbilical cord blood**: there was no significant difference in the median plasma concentration of sEng in the umbilical cord blood of neonates born to mothers with PTL with (n=40) and without (n=40) histologic chorioamnionitis/funisitis [median 6.22 ng/mL, IQR 5.65-7.33 ng/mL vs. median 6.54 ng/mL, IQR 5.62-7.51 ng/mL; p=0.6].

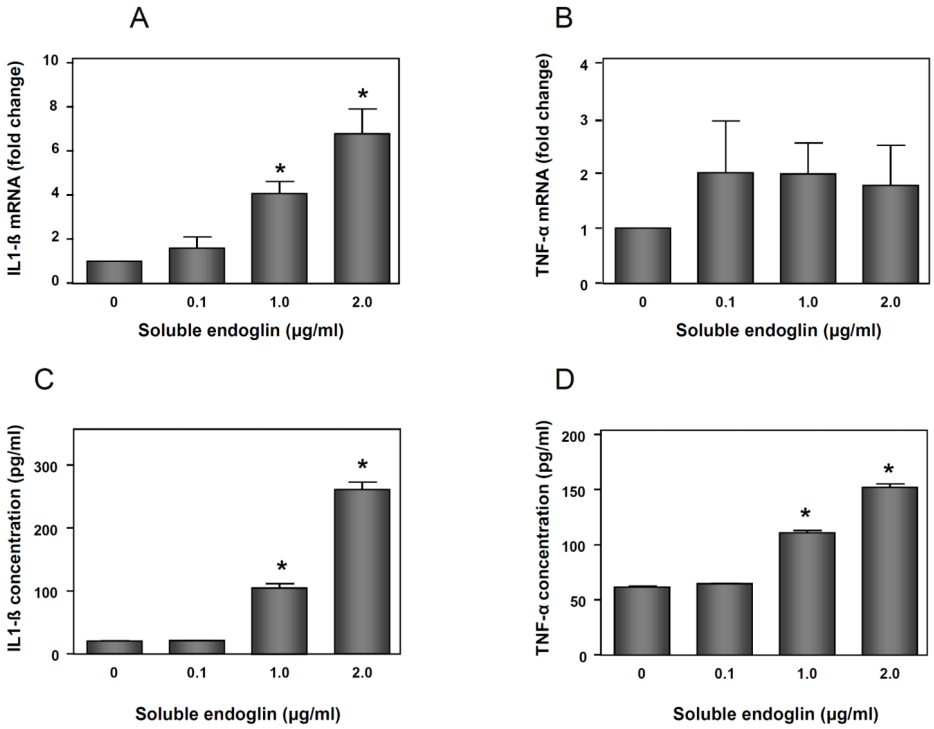

Figure 8. Effects of soluble endoglin (sEng) on U937-derived macrophages

(A) The mean mRNA expression of IL-1β on macrophages was significantly increased after treatment with recombinant sEng at 1 and 2 µg/mL ($p<0.05$ for each). (B) The mean mRNA expression of TNF-α was also increased after treatment with recombinant sEng, but the difference did not reach statistical significance. (C, D) The mean concentration of IL-1β and TNF-α protein in supernatant was significantly increased after treatment of macrophages with recombinant sEng at 1 and 2µg/mL. Data represents mean ± SEM. All the experiments were done in triplicate. The p-values were calculated by Student's t-tests. (*)= $p<0.05$.

Table I

Demographic and clinical characteristics of women in the midtrimester, those at term not in labor, and those in spontaneous labor at term

	Midtrimester (n=20)	*p* [a]	Term no labor (n=44)	Term in labor (n=48)	*p* [b]
Maternal age (years)	37.0 (35.0-38.8)	<0.001	27.0 (21.8-32.8)	22.0 (19.3-26.8)	0.005
GA at amniocentesis (weeks)	16.1 (16.0-17.0)	<0.001	38.5 (38.0-39.0)	38.5 (37.6-39.3)	NS
GA at delivery (weeks)	40.0 (39.0-40.0)	0.001	38.5 (38.0-39.0)	38.5 (37.6-39.3)	NS
Birthweight (g)	3445 (3261-3824)	NS	3250 (3065-3563)	3355 (3078-3548)	NS

Values are expressed as median (interquartile range).

GA, gestational age; NS, not significant.

[a] *p*, comparison between patients in the midtrimester and those at term not in labor.

[b] *p*, comparison between patients at term not in labor and those at term in labor.

Table II

Demographic and clinical characteristics of patients presenting with spontaneous preterm labor with intact membranes.

	PTL without IAI term delivery (n=95)	*p*	PTL without IAI preterm delivery (n=46)	*p* [a]	PTL with IAI preterm delivery (n=40)	*p* [b]
Maternal age (years)	22.0 (19.0-27.0)	NS	23.3 (19.0-30.0)	NS	23.5 (18.8-29.3)	NS
Smoking	15.8 (15/95)	NS	10.9 (5/46)	NS	26.3 (10/38)	NS
Nulliparity	45.3 (43/95)	NS	39.1 (18/46)	0.02	65.0 (26/40)	0.04
GA at amniocentesis (weeks)	31.9 (29.1-33.3)	NS	31.1 (27.5-33.1)	NS	30.6 (27.0-32.9)	NS
GA at delivery (weeks)	38.7 (37.9-39.9)	<0.001	34.3 (32.7-35.6)	<0.001	31.1 (27.3-33.3)	<0.001
Birthweight (g)	3240 (2970-3550)	<0.001	2370 (1783-2700)	<0.001	1655 (910-2118)	<0.001
Birthweight <10th percentile	3.2 (3/95)	NS	6.5 (3/49)	NS	5.0 (2/40)	NS

Values are expressed as percentage (number) or median (interquartile range).

PTL, preterm labor; GA, gestational age; IAI, intra-amniotic infection/inflammation; NS, not significant.

p, comparison between patients PTL who delivered at term and PTL without IAI.

[a] *p*, comparison between patients PTL who delivered preterm without IAI and PTL with IAI.

[b] *p*, comparison between patients PTL who delivered at term and PTL with IAI.

Table III

Demographic and clinical characteristics of patients presenting with preterm prelabor rupture of membranes.

	Preterm PROM without IAI (n=37)	Preterm PROM with IAI (n=37)	*p*
Maternal age (years)	24.0 (20.0-32.5)	30.0 (24.3-37.0)	0.009
Smoking	21.6 (8/37)	22.2 (8/36)	NS
Nulliparity	37.8 (14/37)	16.2 (6/37)	NS
GA at amniocentesis (weeks)	31.6 (28.9-33.8)	30.6 (28.5-32.7)	NS
GA at delivery (weeks)	33.0 (31.1-34.5)	30.9 (28.7-33.5)	0.002
Birthweight (g)	2010 (1655-2265)	1740 (1380-2380)	NS
Birthweight <10th percentile	0 (0/37)	2.8 (1/36)	NS

Values are expressed as percentage (number) or median (interquartile range).

PROM, prelabor rupture of membranes; GA, gestational age; IAI, intra-amniotic infection/inflammation; NS, not significant.