OBSTETRICS

Circulating angiogenic factors in monochorionic twin pregnancies complicated by twin-to-twin transfusion syndrome and selective intrauterine growth restriction

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OBJECTIVE: To determine maternal plasma levels of soluble vascular endothelial growth factor receptor-1 (sVEGFR-1), placental growth factor (PLGF), and soluble endoglin (sEng) in monochorionic diamniotic (MC/DA) twin pregnancies complicated by twin-to-twin transfusion syndrome (TTTS) or selective intrauterine growth restriction (sIUGR).

STUDY DESIGN: A longitudinal cohort study of pregnant women with MC/DA twins who were classified into 3 groups: (1) uncomplicated MC/DA twins (n = 22), (2) TTTS (n = 23), and (3) sIUGR (n = 15). Maternal plasma samples were obtained between 13-20 and 21-28 weeks of gestation and cord blood samples were collected at delivery. Maternal plasma concentrations of sVEGFR-1, PLGF, and sEng, as well as cord blood levels of sVEGFR-1 were measured by enzyme-linked immunoassay.

RESULTS: Maternal plasma levels of sVEGFR-1 and sEng were significantly higher in patients with TTTS at the early and late second trimester compared with normal monochorionic pregnancies (P < .01). In contrast, in the sIUGR group, sVEGFR-1 and sEng levels were significantly higher only at the late second trimester (P < .05). PLGF levels were significantly lower at the early and late second trimester in both TTTS and sIUGR compared with controls (P < .01). Plasma concentrations of sVEGFR-1 were significantly higher among TTTS pregnancies compared with sIUGR at the late second trimester (P = .027). Cord blood levels of sVEGFR-1 were significantly higher in the smaller intrauterine growth restricted twin compared with the normal cotwin.

CONCLUSION: Monochorionic pregnancies complicated by TTTS and sIUGR are characterized by decreased angiogenic activity. The disparity in severity of the antiangiogenic state between TTTS and sIUGR suggests that these 2 conditions may represent a continuum.

Key words: angiogenic factors, monochorionic twins, PLGF, sEng, sIUGR, sVEGFR-1, TTTS

M onochorionic twin pregnancies are associated with increased perinatal morbidity and mortality compared with their dichorionic counterparts and singleton pregnancies.1,2 The increased risk of monochorionic pregnancies is largely attributable to the presence of vascular anastomoses connecting the 2 fetal circulations. The intertwin anastomoses are nearly always present and account for a range of pregnancy complications, including twin-to-twin transfusion syndrome (TTTS), twins anemia polycythemia sequence (TAPS), and neurologic injury to the surviving twin in the event of intrauterine demise of its cotwin.3,4 TTTS is the result of an unbalanced transfusion of blood across placental vascular anastomoses from one twin (donor) to the other (recipient).5,6 However, even though almost all monochorionic twin placentas have vascular anastomoses, TTTS develops only in 10-15% of these pregnancies.8 Therefore, although the presence of vascular anastomoses is mandatory for the development of TTTS, it’s mere presence is not sufficient and other additional factors may play a role in the pathophysiology of this condition.

In addition, the single monochorionic placenta has to nourish 2 fetuses and is often not equally shared, which may lead to growth restriction and severe discordant birthweight.9 Selective intrauterine growth restriction (sIUGR) affects about 12-25% of monochorionic twin pregnancies4 and is increasingly recognized as an important complication of monochorionic twin pregnancies. Indeed, sIUGR is associated with an increased fetal and neonatal mortality and morbidity.10,11 The ability to predict monochorionicity-associated complications of pregnancy is quite limited and is mainly based on first trimester sonographic markers.12,13 Moreover, the differentiation between TTTS and sIUGR could be challenging as both might be manifested by discordant growth, oligohydramnios in one sac and...
absent or reverse end-diastolic velocity in the umbilical artery of the small twin. The importance of distinctions between these 2 conditions stems from the fact that the management of these complications is different: laser coagulation of the vascular anastomoses is the treatment of choice versus conservative management with close surveillance or selective umbilical cord occlusion in cases of high risk of in-utero fetal demise, although laser coagulation of anastomoses has also been used in certain cases.

Angiogenesis plays a central role in normal placental development. It has been shown that angiogenic factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) and 2 antiangiogenic peptides produced by the placenta, soluble vascular endothelial growth factor receptor-1 (sVEGFR-1) and soluble endoglin (sEng), contribute to the pathogenesis of preeclampsia as well as IUGR, both associated with aberrant placenta.

Because there is no clear explanation why only certain monochorionic twins are complicated by TTTS or sIUGR, new mechanisms, which play a role in the pathophysiology of these conditions, must be explored. We hypothesized that altered angiogenesis mediated by sVEGFR-1, sEng and PLGF might be the mechanism linking abnormal placentation with pregnancy complications of monochorionic twins.

The aim of this study was to determine maternal and fetal plasma levels of sVEGFR-1, PLGF, and sEng in monochorionic diamniotic (MC/DA) twin pregnancies complicated by TTTS or sIUGR compared with uncomplicated monochorionic twins.

Materials and Methods
This was a prospective cohort study of patients with MC/DA twin pregnancies who were enrolled between November 2010 and May 2012 at a single tertiary center. The study was approved by the local institutional ethics committee, and all patients provided a written informed consent. The patients were classified into 3 groups: (1) uncomplicated monochorionic pregnancies (n = 22), (2) TTTS (n = 23), and (3) sIUGR (n = 15). All patients had a first trimester ultrasound, which confirmed the diagnosis of monochorionicity and established an accurate gestational age. The diagnosis of TTTS was based on the internationally accepted ultrasound criteria: an MC twin pregnancy with polyhydramnios of ≥8 cm deepest vertical pocket in the recipient (or ≥10 cm from 20 weeks of gestation onwards) and oligohydramnios of ≤2 cm deepest vertical pocket in the donor. Selective IUGR was defined as an estimated fetal weight below the 10th percentile in 1 twin and estimated fetal weight discordance of 25% or greater.

Patients with a combined pathology of TTTS and sIUGR were classified in the TTTS group. The inclusion criteria for uncomplicated monochorionic pregnancies included: (1) appropriately grown fetuses; (2) estimated fetal weight difference of less than 25%; (3) normal amniotic fluid volumes; (4) normal Doppler velocimetry in the umbilical arteries; and (5) similar measurements of the middle cerebral artery-peak systolic velocity among both twins. Patients with chronic hypertension and pregestational diabetes, as well as pregnancies complicated with congenital anomalies or chromosomal abnormalities or intrauterine fetal death of 1 of the twins at presentation were excluded. Patients whose pregnancies were complicated by severe TTTS (defined as TTTS stage ≥2) were treated by fetoscopic laser ablation (n = 20). Patient with TTTS stage 1 (n = 3) were managed conservatively. Demographic data, ultrasound findings, and perinatal outcomes were entered into a computerized database. Serial samples of peripheral blood were obtained throughout pregnancy starting at the patient’s first visit to our fetal medicine clinic and thereafter every 6 weeks. All the maternal samples used for analysis were taken before laser...
treatment or selective termination. Cord blood samples were obtained at delivery. Blood samples were collected in tubes containing EDTA, centrifuged at 4°C for 10 minutes and stored at −70°C until further analysis. Maternal plasma levels of sVEGFR-1, sEng, and PLGF and cord blood levels of sVEGFR-1 were determined by enzyme-linked immunoassays (R&D Systems, Minneapolis, MN). All samples were assayed in duplicate at the same time using the same standard curve to minimize interassay variation. The calculated interassay coefficients of variation for sVEGFR-1, sEng, and PLGF were 7.4%, 7.1%, and 8.3%, respectively. The calculated intraassay coefficients of variation for sVEGFR-1, sEng, and PLGF were 2.4%, 2.6%, and 4.8%, respectively.

Normality of the data was tested using Kolmogorov-Smirnov test. Because the data did not fit a normal distribution, values of the factors tested are presented as median and interquartile range. The 3 groups were compared using Kruskal-Wallis test, and comparison of continuous variables between every 2 groups was conducted using Mann-Whitney U test. The χ² test was used for comparison of categorical variables. Significance was accepted at P < .05. Statistical analyses were conducted using the IBM Statistical Package for the Social Sciences (IBM SPSS v.19; IBM Corporation Inc, Armonk, NY).

Results
Sixty patients were included in the study; 22 were uncomplicated monochorionic twins, 23 were diagnosed with TTTS, and 15 with sIUGR. The demographic and clinical characteristics of the study population are shown in Table 1. Of the 23 patients with TTTS included in this study, 3 had a stage 1 disease and were followed conservatively, 12 had a stage 2 disease, and 8 presented with TTTS stage 3-19 of them underwent fetoscopic laser ablation and 1 patient had selective termination. Four of the patients in the TTTS group had a combined pathology of TTTS and sIUGR. Twelve of the 15 patients with sIUGR had abnormal Doppler in the umbilical artery; 10 had persistent AEDV, and 2 had intermittent AEDV. Five of the patients in this group underwent selective termination with radiofrequency ablation. All 3 groups were comparable in terms of maternal age, parity, maternal pregestational body mass index, and the rate of smokers. None of the pregnancies in our cohort were complicated by preeclampsia. As expected, patients with TTTS and sIUGR delivered earlier compared with the uncomplicated MC twins (32.2, 32.5, and 36.2 weeks of gestation respectively, Kruskal-Wallis P = .009).

The gestational age at blood sampling did not differ among the 3 groups (Table 1).

We have divided the time frame in which maternal blood samples were analyzed into 2: early second trimester (13-20 weeks of gestation) and late second trimester (21-28 weeks of gestation).

Patients with TTTS had significantly higher median plasma levels of sVEGFR-1 (3835.2 pg/mL vs 1359.9 pg/mL, respectively, P < .01, Figure 1, A), and higher median plasma levels of sEng (22.8 vs 7.1 ng/mL, respectively, P < .01, Figure 2, B) at 21-28 weeks of gestation. In contrast, median plasma levels of sVEGFR-1 (1671.4 vs 1359.9 pg/mL, respectively, P = .13 Figure 1, A), and sEng (9.6 vs 6.6 ng/mL, respectively, P = .27, Figure 2, A) did not differ significantly between patients with sIUGR and controls at 13-20 weeks of gestation. However, at 21-28 weeks of gestation patients with sIUGR had significantly higher median plasma levels of sVEGFR-1 and sEng compared with normal MC twins (3237.2 pg/mL vs 1927 pg/mL, P = .035; 18.8 ng/mL vs 7.1 ng/mL, P < .01, respectively, Figures 1, B, and 2, B).

Both patients with TTTS and sIUGR had significant lower median plasma levels of PLGF compared with normal MC twins at the early second trimester (140.3 and 281.9 vs 514.8 pg/mL, P < .01, respectively, Figure 3, A) as well as at the late second trimester (263.7 and 350.1 pg/mL vs 956.8 pg/mL, P < .01, respectively, Figure 3, B).

Comparison between TTTS and sIUGR: the median plasma levels of sVEGFR-1 were higher among patients
with TTTS compared with patients with sIUGR although this difference was significant only at the late second trimester (6068.7 vs 3237.2 pg/mL, \( P = .027 \), Figure 1, B).

Maternal plasma levels of sVEGFR-1, sEng, and PLGF as a function of gestational age are shown in Figure 4. In 2 of our TTTS cases, maternal blood was sampled before the development of TTTS. In the first patient, blood was initially sampled at 15 weeks of gestation when only amniotic fluid discordance appeared on ultrasound and subsequently developed severe TTTS at 20 weeks of gestation. In the second patient, blood samples were initially collected at 18 weeks of gestation followed by development of severe TTTS at 21 weeks of gestation. In both patients, sVEGFR-1 levels were higher and PLGF levels were lower at the first blood sample 5 and 3 weeks before the development of TTTS.

Among newborns, cord blood analysis revealed significantly higher median plasma levels of sVEGFR-1 among the IUGR twins compared with the normal cotwins (458.2 pg/mL vs 100.7 pg/mL, \( P < .01 \), Figure 5). In contrast, no differences were found between the twins of normal MC pregnancies or between the former donors and former recipients of TTTS pregnancies. Six of the 8 TTTS pregnancies whose cord blood was analyzed for sVEGFR-1 level had laser ablation during pregnancy.

**COMMENT**

Our study have demonstrated that TTTS pregnancies are characterized by increased maternal plasma levels of sVEGFR-1 and sEng and decreased maternal plasma levels of PLGF both at the early and late second trimester compared with normal MC twins. In contrast, in pregnancies complicated by sIUGR, sVEGFR-1, and sEng were significantly increased only at the late second trimester. Moreover, maternal plasma levels of sVEGFR-1 were significantly increased among TTTS pregnancies compared with sIUGR. Finally, we have shown elevated cord blood levels of sVEGFR-1 among the small IUGR twins compared with their normal cotwins but no differences between the donor and recipient following pregnancies complicated by TTTS.

TTTS is thought to result from an unbalanced intertwin blood flow through placental vascular anastomoses. At least 1 unidirectional arteriovenous anastomosis is required for the development of TTTS, whereas the presence of an arterioarterial anastomosis seems...
to be protective.\textsuperscript{6,7} However, a large body of evidence strongly suggests that the pathophysiology of TTTS cannot be attributed solely to vascular anastomoses because almost all monochorionic placentas have vascular anastomoses but only 10–15\% develop TTTS.\textsuperscript{8} Therefore, other pathologic processes may play a role in the pathophysiology of this disease.\textsuperscript{4} Our findings indicate that TTTS is an anti-angiogenic state, supporting previous reports, which have showed maternal angiogenic activity to be decreased in TTTS.\textsuperscript{25,26} Kusanovic et al\textsuperscript{25} have collected blood samples from 16 patients with TTTS between 16–26 weeks of gestation and found elevated levels of sVEGFR-1 and sEng and decreased levels of PLGF compared with women with monochorionic twins without TTTS. Similarly, Fox et al\textsuperscript{26} reported on increased plasma levels of sVEGFR-1 as well as increased sVEGFR-1/PLGF ratio among patients with TTTS, but no differences were found in PLGF levels between TTTS pregnancies and uncomplicated MC twins. Fox et al\textsuperscript{26} have showed a transient increase in sVEGFR-1/PLGF ratio after laser ablation followed by reduction to below basal levels by 1 week. Despite this consistent observation of decreased angiogenesis in pregnancies complicated by TTTS, the initiating event leading to increased placental secretion of sVEGFR-1 and sEng is still unknown.

Our study is the first to show differences in the levels of angiogenic factors between TTTS and sIUGR with increased maternal plasma levels of sVEGFR-1 in pregnancies complicated by TTTS compared with sIUGR. The anti-angiogenic state became apparent in sIUGR pregnancies only at the late second trimester. The results of this study strongly suggest that TTTS is associated with intense severity of antiangiogenic state compared with sIUGR. Both temporal differences, ie, earlier evidence of antiangiogenic state, and higher concentrations of antiangiogenic factor in the TTTS group support this hypothesis. Even though TTTS and sIUGR have strict definitions and are regarded as 2 distinct pathologies, the findings reported herein suggest that these 2 conditions may represent a continuum. In other words, assuming that antiangiogenic state plays an important role in the pathophysiology of TTTS and sIUGR, sIUGR may be regarded as a forme fruste of TTTS. However, more evidence is needed to establish this hypothesis.

Growth in monochorionic twins is determined by the division of the single placenta between the twins as well as by the vascular anastomoses.\textsuperscript{9,27} All the sIUGR cases included in our study were characterized by early-onset discordant growth, which typically have an unequally shared placenta with large anastomoses.\textsuperscript{27} Because vascular anastomoses also influence growth in monochorionic twins, unbalanced net arteriovenous transfusion as occurs in TTTS may result in growth restriction of the donor twin. Therefore, each of these 2 conditions could evolve into the other resulting in a combined pathology of TTTS and sIUGR.\textsuperscript{27,28} Moreover, the clinical distinction between these pathologies is not always clear. Therefore, it is possible that maternal plasma levels of angiogenic factors may assist in differentiating between sIUGR and TTTS pregnancies or even predict later development of TTTS in pregnancies complicated by sIUGR. In contrast to our findings, Kusanovic et al\textsuperscript{25} did not observe any differences in maternal plasma levels of sVEGFR-1, sEng, and PLGF between MC twins with and without IUGR. The fact that most of our sIUGR patients had AEDV in the umbilical artery as well as early onset of growth restriction might explain the differences between these 2 studies.

Finally, cord blood analysis revealed increased sVEGFR-1 levels in the IUGR twin compared with the normal cotwin. This observation is in accordance with previous studies on MC twins with sIUGR showing increased expression of sVEGFR-1 as well as endoglin in the IUGR twin placenta compared with the normal cotwin’s placenta.\textsuperscript{22,23} However,
unbalanced net arteriovenous transfusion may have also contributed to the difference in cord blood levels of sVEGFR-1 between the IUGR twin and the normal cotwin. Despite previous report showing that VEGFR-1 mRNA is overexpressed in the villi of the donor in some cases of TTTS,29 we did not find any differences in cord blood levels of sVEGFR-1 between the donor and the recipient, perhaps because most of these cases were treated by laser ablation during pregnancy with resolution of the condition.

As a tertiary fetal therapy center, many of the patients were referred to us only when TTTS or sIUGR were suspected and therefore, in most cases, the samples were collected when the diagnosis was already established. Hence, the current study cannot determine whether these factors can predict development of TTTS or sIUGR in MC twins early in pregnancy, and whether the antiangiogenic state is a cause or consequence of TTTS. Interestingly, in 2 of our cases the alteration in the maternal plasma levels of sVEGFR-1 and PLGF preceded the onset of TTTS by 3-5 weeks. Therefore, further studies are needed to evaluate the predictive value of these factors in MC pregnancies. It is possible that by measuring maternal circulatory levels of sVEGFR-1, sEng, and PLGF early in gestation, we will be able to detect which of the MC pregnancies are prone to develop TTTS or sIUGR later in gestation. Furthermore, measurement of these factors might be proven useful clinically in differentiating between TTTS and sIUGR.

To conclude, our study demonstrates that TTTS is characterized by an antiangiogenic state, in accordance with previous reports. Although antiangiogenic state becomes apparent only at the late second trimester in pregnancies complicated by sIUGR, TTTS is characterized by antiangiogenic state that is earlier and more intense in comparison. The disparity in severity of the antiangiogenic state between TTTS and sIUGR suggests that these 2 conditions may represent a continuum. Further studies are required to determine the predictive value of angiogenic and antiangiogenic factors in MC pregnancies as well as their importance in early distinction between sIUGR and TTTS.

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