Table 3
Antiphospholipid antibodies as risk factors for premature delivery (<37 and <34 weeks' gestation) determined by univariate analysis).

aPL	Prevalence of premature delivery (<37 weeks) in positive population	*P*-value	Relative risk (95% CI)	Prevalence of premature delivery (<34 weeks) in positive population	*P*-value	Relative risk (95% CI)
LA	55.6%	<0.0001	6.6 (2.2–20.1)	0		
IgG aCL	23.1%			7.7%		
IgM aCL	7.7%			0		
IgA aCL	16.7%			0		
IgG aPS/PT	50.0%			0		
IgM aPS/PT	0			0		
IgG aPE	18.2%			12.5%	0.004	10.1 (2.7–37.5)
Any aPL	17.1%	0.011	2.1 (1.1–4.0)	5.4%	0.020	4.5 (1.4–14.1)
Multi-positive	50.0%	0.041	5.8 (1.04–31.9)	0		
Double-positive (LA and aCL)	66.7%	0.022	7.7 (1.3–46.6)	0		

aPL, antiphospholipid antibody; LA, lupus anticoagulant; aCL, anticardiolipin antibody; aPS/PT, phosphatidylserine-dependent antiprothrombin antibody; aPE, kininogen-dependent antiphosphatidylethanolamine antibody; PIH, pregnancy-induced hypertension.

aPE, and any aPL increased the risk of PIH, while IgG aPE, any aPL, multi-positive, and double-positive, increased the risk of severe PIH (Table 2). We found weak associations between pre-eclampsia and aPE IgG (RR 5.9, 95% CI 1.3–27.1), but with borderline significance ($p = 0.052$).

Table 3 presents risk factors for PD at <37 GW and PD at <34 GW. LA, any aPL, multi-positive, and double-positive, increased a risk of PD at <37 GW, while IgG aPE and any aPL increased the risk of PD at <34 GW. LA (RR 4.6, 95% CI 1.5–13.9, $p = 0.002$) increased a risk of LBW. None of the aPLs was associated with a risk of FGR <10th percentile, FGR <−1.5 S.D., or fetal loss.

A multiple logistic regression model with adjustment for confounding factors demonstrated that aPLs were risk factors for serious adverse pregnancy outcomes including PIH, severe PIH, PD, and LBW (Table 4).

To determine whether a positive test for any of the two aPLs effectively predicted the most serious adverse pregnancy outcome (i.e., PIH and severe PIH), we searched for a combination of two types of aPLs. According to Benjamini and Hochberg's correction to control the false discovery rate in a multiple comparison, the only combination of IgG aPE plus IgG aCL measurement was found to be a significant combination that predicted PIH with 19.4% sensitivity and 97.4% specificity (OR 10.6,

Table 4
Antiphospholipid antibodies as risk factors for serious adverse pregnancy outcomes determined by multivariate analysis.

Adverse pregnancy outcome	aPL	Odds ratio	95% CI
PIH	IgG aCL	11.4	2.7–47.6
	IgG aPE	8.3	2.4–28.6
	Any aPL	5.5	2.3–13.5
Severe PIH	IgG aPE	20.4	4.5–90.9
	Any aPL	8.1	2.2–29.4
	Multi-positive	143	9.8–1000
	Double-positive (LA and aCL)	250	11.1–1000
Premature delivery (<37 weeks)	LA	11.0	2.8–43.5
	Any aPL	2.3	1.1–4.4
	Multi-positive	11.6	1.5–90.9
	Double-positive (LA and aCL)	22.2	1.9–250
Premature delivery (<34 weeks)	IgG aPE	12.7	3.1–50.0
	Any aPL	4.5	1.4–14.9
Low birth weight	LA	8.0	2.1–31.3
	Double-positive (LA and aCL)	13.7	1.2–167

aPL, antiphospholipid antibody; PIH, pregnancy-induced hypertension; aCL, anticardiolipin antibody; aPE, kininogen-dependent antiphosphatidylethanolamine antibody; LA, lupus anticoagulant.

95% CI 4.0–28.6, $p < 0.0001$). Similarly, the combinations of IgG aPE plus IgG aCL, and IgG aPE plus LA predicted severe PIH with 30.8% sensitivity and 99.2% specificity (OR 17.5, 95% CI 4.7–66.7, $p < 0.0001$), and with 30.8% sensitivity and 99.2% specificity (OR 22.2, 95% CI 5.4–909, $p < 0.0001$). Any combination of three or more kinds of aPL measurement did not significantly improve the sensitivity and specificity for the prediction of PIH or severe PIH. When one of the lifestyle-related factors, high BMI ($\geq 25\,kg/m^2$), was added to the aPL combination of IgG aPE plus IgG aCL, the presence of high BMI or a positive test for the aPLs predicted severe PIH with higher sensitivity (53.8%) and specificity (99.4%; OR 11.2, 95% CI 3.4–37.0, $p < 0.0001$).

4. Discussion

In this study, we found that lifestyle-related confounding factors were significantly associated with the risks of a serious adverse pregnancy outcome, including high BMI with PIH, severe PIH, and pre-eclampsia; cigarette smoking with FGR and fetal loss; drinking alcohol with PD and fetal loss; and primiparity with PIH and multiparity with FGR. It is well documented that obesity, primiparity, and age ≥35 years are risk factors for PIH (Poole, 1997) and pre-eclampsia (ACOGCOP, 2002; Lain and Roberts, 2002). It has also been reported that smoking increases the risks of FGR and fetal loss (Ness et al., 1999; Lindbohm et al., 2002) and drinking alcohol increases the risk of FGR, PD (Windham et al., 1995), and fetal loss (Kesmodel et al., 2002). Our findings are comparable to the aforementioned, suggesting that our study participants had little or no deviation in lifestyle from that of the standard population of pregnant women. In this study, multiparity was related to FGR <10th percentile, but not to FGR <−1.5 S.D. We speculate that in our participants, some lifestyle factors, such as diet for multiparas who were city dwellers, might affect fetal growth. By adjusting for the confounding factors described above, we determined that positive tests for aPL measurements in early pregnancy were risk factors for the occurrence of PIH, severe PIH, PD, and LBW later in the pregnancy.

We found that IgG aCL, IgG aPE, and multi-positive aPL was a risk factor for PIH or severe PIH; and that double-positive aPL (LA and aCL) was a risk factor for severe PIH. To the best of our knowledge, this is the first evidence regarding the association between the multi-/double-positive aPL and PIH. Similarly, recent studies have suggested that the multi-positive test is associated with a more severe course of APS disease, increasing significantly the rate of thrombosis (Detkova et al., 1999; Lee et al., 2003a; Obermoser et al., 2003, 2004). Thus, it is likely that multi-/double-positive aPL predicts a higher risk of PIH and severe PIH as well. Pregnant women with multiple/double aPL should be more carefully managed during pregnancy.

In the present study, LA and double-positive aPL were associated with PD at <37 GW and LBW. In this study design, there was the limitation that the physicians knew the results of LA and aCL measurements in women who had a history of thromboembolism or RPL because these aPLs are included in the laboratory findings of the APS criteria (Wilson et al., 1999; Miyakis et al., 2006). The knowledge of the presence of these aPLs may have influenced the physician in favor of an early pregnancy termination. The possibility of this bias cannot be excluded.

Little is known about the relationship between thromboembolism/adverse pregnancy outcome and aPE; however, recent studies have reported that there are associations. More specifically, aPE was frequently detected in patients with unexplained recurrent early fetal loss, mid-to-late fetal loss, unexplained thrombosis, systemic lupus erythematosus, heart valvulopathies, and livedo reticularis (Gris et al., 2000; Sanmarco et al., 2001; Balada et al., 2001; Yamada et al., 2003; Sugi et al., 2004). In the current study, it was demonstrated by a multivariate analysis that aPE increased the risk of PIH, severe PIH, and PD at <34 GW. All women who developed severe PIH with aPE ended up undergoing induced PD at <34 GW by cesarean section (data not shown). We measured the kininogen-dependent antiphosphatidylethanolamine antibody that probably binds to kininogen as a cofactor (Sugi et al., 1999). The kallikrein–kinin system is involved in blood pressure control and angiogenesis. Tissue kallikrein cleaves low-molecular-weight kininogen substrate to produce the vasodilator Lys-bradykinin, whereas plasma kallikrein forms bradykinin (BK) from high-molecular-weight kininogen (HK). Kininogen-deficient rats are susceptible to the development of salt-induced hypertension (Majima et al., 1994), and the in vivo angiogenesis is suppressed (Hayashi et al., 2002). The pro-angiogenic effect of BK and HK has been demonstrated in both in vitro and in vivo studies (Guo and Colman, 2005). Therefore, we assume that aPE pathophysiologically causes impairment of fetoplacental angiogenesis and vessel development, which subsequently may predispose women to PIH. Alternatively, disruption of the kininogen cascade in the kallikrein–kinin system may reduce vasodilator production and cause a hypertensive disorder. Recently, a multicenter study demonstrated that aPE, but not LA or aCL, was closely associated with thrombosis, having

the highest odds ratio (Sanmarco et al., 2007). It is likely that the thrombotic insult is causally associated with PIH.

Among patients with systemic autoimmune disease, it has been demonstrated that IgG aPS/PT, but not antiprothrombin antibody (aPT-A), is related with a high specificity to thromboembolism and to the presence of LA (Atsumi et al., 2000). Our group has assessed a wide variety of aPLs in women with RPL and demonstrated an association between IgG aPS/PT and second-trimester fetal death (Yamada et al., 2003). However, neither aPS/PT nor aPT-A has been evaluated in a prospective study on pregnant women. For the first time, the present study assessed aPS/PT in relation to serious adverse pregnancy outcomes. The prevalence of PD at <37 GW or LBW among women who tested positive for IgG aPS/PT was as high as 50%, but without statistical significance.

We searched for the aPL combination with the highest ability to predict the most serious adverse pregnancy outcome, i.e., PIH and severe PIH. It was found that the combinations of IgG aPE plus IgG aCL, and IgG aPE plus LA measurements predicted PIH or severe PIH with relatively high specificity, but with low sensitivity. If one of the lifestyle-related factors, high BMI, was considered in addition to the aPL combination of IgG aPE plus IgG aCL, the presence of high BMI or a positive test for any of the two aPLs predicted severe PIH, with an increased sensitivity (53.8%) and a high specificity (99.4%). This suggests that, with a combination of high BMI, aPE IgG, and aCL IgG measurements during early pregnancy screening, approximately 50% of women who later develop severe PIH might be detected.

We reanalyzed our data using 95th percentile cut-off values for aPLs instead of the 99th percentile values. Overall, the latter more specifically detected serious adverse pregnancy outcomes than the former and higher odds ratios were exhibited (data not shown). When we commenced this study, the laboratory criteria for APS did not include IgG and IgM aβ_2GPI (Wilson et al., 1999). These aPLs should be prospectively assessed in future in relation to pregnancy complications, because amendments to the APS criteria have recommended these measurements (Miyakis et al., 2006). Serious adverse pregnancy outcomes evaluated in the current study are naturally polygenetic-multifactorial diseases. Not only aPLs, but also genetic background, other lifestyle-related factors, management policies of doctors and facilities, and any maternal complications could affect these outcomes. Under the circumstances, we demonstrated that specific aPLs and their combination increased the risks of PIH, severe PIH, PD, and LBW.

For the first time, we determined that aPE increased the risk of PIH, severe PIH, and PD at <34 GW using a multivariate analysis. If a woman has a history of thrombosis, mid-trimester fetal loss, PD at <34 GW, pre-eclampsia or recurrent spontaneous abortion, blood screening of LA, aCL, and aβ_2GPI measurements can be recommended, because these aPLs are already included in the APS laboratory criteria (Miyakis et al., 2006). If a woman with such a history tested negative for these aPLs, aPE should be further assessed. The preventive efficacy of anticoagulation therapy such as low-dose aspirin and heparin in pregnant women with aPE and this history should be assessed in future prospective studies.

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ORIGINAL ARTICLE

Specific ultrasound findings associated with fetal chromosome abnormalities

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ABSTRACT **Cytogenetic amniocentesis (CA) has been performed as a reliable prenatal diagnostic method for decades. The aims of the present study were to reveal the frequency of fetal chromosome abnormalities according to medical indications of CA, and to assess the risks of specific abnormal ultrasound findings. Data on chromosome karyotypes of fetuses from 5043 Japanese mothers were collected. Group I comprised 4626 fetuses whose mothers underwent CA due to a variety of parental reasons. Group II comprised 417 fetuses whose mothers underwent CA due to fetal abnormality, abnormality of amniotic fluid volume and fetal growth restriction. The frequency of chromosome abnormalities in Group II (17.7%) was significantly higher than in Group I (1.8%). The frequencies of chromosome abnormalities in Group II singleton fetuses with fetal abnormality, polyhydramnios and fetal growth restriction were 21.5, 22.9 and 19.6%, respectively. By multivariate analyses, we found that cystic hygroma (odds ratio 5.6, 95% CI 2.7–11.6), abnormal extremity (5.0, 1.7–14.4) and cardiovascular abnormality (3.3, 1.1–10.1) were significant variants associated with fetal chromosomal abnormalities. Information revealed in the present study constitutes a beneficial reference for genetic counseling.**

Key Words: amniocentesis, amniotic fluid volume, chromosome abnormality, fetal abnormality, ultrasound

INTRODUCTION

Cytogenetic analysis following amniocentesis (i.e. cytogenetic amniocentesis [CA]), has been performed as a reliable prenatal diagnostic method in women with an increased risk of giving birth to a child with a chromosome abnormality. Medical indications of CA include a wide variety of status, such as advanced maternal age, chromosome abnormality in the parents, abnormal value of maternal serum markers, gestational history of recurrent spontaneous abortion and a child with chromosome abnormality, fetal abnormality or growth restriction detected by prenatal ultrasound examinations.

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Evidence-based genetic counseling for pregnant women who consider undergoing CA is necessary because CA has a risk of rupture of the membranes and fetal anomalies cannot usually be cured *in utero*.

In the present study, data of fetal chromosome karyotypes obtained from 5043 consecutive Japanese patients who underwent CA at one medical center of prenatal diagnosis were collected. We assessed the frequency of chromosome abnormalities according to medical indications of CA, and by a univariate analysis we determined ultrasound findings and maternal factors that were related to fetal chromosome abnormalities. A multivariate analysis was performed to determine independent ultrasound findings associated with fetal chromosome abnormalities. Results reported in the study constitute an important reference for discreet genetic counseling prior to CA.

MATERIALS AND METHODS

Over a period of 24 years, 5293 Japanese pregnant women underwent CA with written informed consent after pre-test counseling at Hokkaido University Hospital. Chromosome karyotypes of fetuses from 5043 mothers were obtained successfully.

The data of fetal chromosome karyotypes were divided into two groups. Group I comprised 4626 fetuses whose mothers underwent CA due to a variety of parental reasons, including advanced maternal age, chromosome abnormality in the parents, abnormal value of maternal serum markers, gestational history of recurrent spontaneous abortion and a child with chromosome abnormality or other, but not fetal abnormality, abnormality of amniotic fluid volume or fetal growth restriction. Group II comprised 417 fetuses whose mothers underwent CA due to fetal abnormality, abnormality of amniotic fluid volume and fetal growth restriction detected by prenatal ultrasound examination. Group I mothers underwent CA early in the second trimester, and Group II mothers in the second or third trimesters.

Frequencies of chromosome abnormalities were compared between Groups I and II, and compared according to medical indications for CA in Group I. We determined ultrasound findings and maternal factors that were associated with fetal chromosome abnormalities. Statistical differences were analyzed by χ^2 test (d.f. = 1). Fisher's exact test was used when an observed number was ≤ 5. A P-value of <0.05 was considered statistically significant. Twenty-three fetuses from multiple pregnancies in Group II were excluded from further analyses because multiple pregnancies affect amniotic fluid volume and fetal growth. Among Group II singleton fetuses, a

stepwise method in multivariate analyses was applied to select significant variants for factors that were related to chromosome abnormality by univariate analyses. A multiple logistic model was applied to evaluate odds ratios (OR) and 95% confidence intervals (95% CI). Univariate and multivariate analyses were conducted with a statistical analysis system package (SAS version 9.1; SAS Institute Japan, Tokyo, Japan).

RESULTS

Frequencies of chromosome abnormality in Groups I and II are shown in Table 1. Frequencies of trisomy 21, trisomy 18, trisomy 13, 45X, deletion or duplication, and others in Group II were significantly higher than in Group I. Balanced type translocation and small Y were not considered chromosome abnormalities.

Table 2 shows the frequencies of chromosome abnormalities and trisomy 21 according to medical indications for CA in Group I. Advanced maternal age was the most frequent indication in Group I. Frequencies of overall chromosome abnormalities and trisomy 21 in women aged 40 or over were significantly higher than in women aged 35–39, as well as in the remaining group.

Women with an indication of chromosome abnormality in parents demonstrated a significantly higher frequency of fetal chromosome abnormality compared with the remaining group (Table 2). In 71 women with an indication of chromosome abnormality in parents, 37 couples with reciprocal translocation, 13 Robertsonian translocations, four inversions, four duplications, three deletions, seven mosaicism of 45X/46XX, one 46XX/47XXX, one 46XY/47XXY, and one 47XXX were included. In this group, 23 of 71 fetuses were found to have the same balanced type translocations as their parents; these translocations were considered normal chromosome karyotypes.

In 509 Group I women who had a previous child with a chromosome abnormality, we investigated the recurrence frequency of fetal chromosome abnormalities. Fetuses with any chromosome abnormality were found in six (1.6%) of 378 women aged 34 or under, and in one (0.8%) of 131 women aged 35 or over. Among women

Table 1 Frequency of chromosome abnormalities in Groups I and II

Chromosome abnormality	Number (%) Group I ($n = 4626$)	Group II ($n = 417$)
Trisomy 21	24 (0.5)	24 (5.8)*
Trisomy 18	9 (0.2)	21 (5.0)*
Trisomy 13	2	3 (0.7)**
XYY plus trisomy 18	0	1 (0.2)
45X	0	8 (1.9)*
XXY	6 (0.1)	1 (0.2)
XYY	2	0
69XXY	0	1 (0.2)
Deletion or duplication	16 (0.3)	5 (1.2)***
Mosaicism with 45X	4 (0.1)	0
Other mosaicism	12 (0.3)	1 (0.2)
Others	9 (0.2)	9 (2.2)*
Total	84 (1.8)	74 (17.7)*

*$P < 0.0001$; **$P < 0.01$; ***$P < 0.05$.

Table 2 Frequency of chromosome abnormalities and trisomy 21 in Group I according to medical indications for cytogenetic amniocentesis

Medical indication		Number (%)	Frequency of chromosome abnormality (%)	Frequency of trisomy 21 (%)
Advanced maternal age (years)	34	32	0	0
	35–39	2116 3035 (65.6)	29 (1.4) 60 (2.0)	4 (0.2) 17 (0.6)
	40≦	887	31*† (3.5)	13**‡ (1.5)
Previous child with chromosome abnormality		509 (11.0)	7 (1.4)	2 (0.4)
Chromosome abnormality in parents		71 (1.5)	4§ (5.6)	0
Chromosome abnormality in family		101 (2.2)	1 (1.0)	1 (1.0)
Previous child with malformation		154 (3.3)	2 (1.3)	0
Anxiety	X-ray exposure	10	1	1
	drug exposure	41 119 (2.6)	0 1 (0.8)	0 1 (0.8)
	Other	68	0	0
Inherited disease		55 (1.2)	1 (1.8)	0
Metabolic disease		24 (0.5)	0	0
Recurrent spontaneous abortion		60 (1.3)	0	0
Assisted reproduction		200 (4.3)	2 (1.0)	0
Abnormal value of maternal serum markers		225 (4.9)	3 (1.3)	3 (1.3)
Other		73 (1.6)	0	0
Total		4626 (100)	81 (1.8)	24 (0.5)

*$P < 0.05$ (≧40 *vs* 35–39); **$P < 0.0001$ (≧40 *vs* 35–39); †$P < 0.0001$ (≧40 *vs* the remaining group); ‡$P < 0.0001$ (≧40 *vs* the remaining group); §$P < 0.05$ (chromosome abnormality in parents *vs* the remaining group).

with a previous child with trisomy 21, fetuses with a chromosome abnormality were found in five (1.9%) of 269 women aged 34 or under, and in one (1.2%) of 85 women aged 35 or over. Among women with a previous child with trisomy 18, fetuses with a chromosome abnormality were found in one (2.8%) of 36 women aged 34 or under, and in none of the 15 women aged 35 or over. Among women with a previous child with trisomy 13 or 45X, no fetuses with a chromosome abnormality were found. We then investigated recurrence frequency of the same chromosome abnormalities. The recurrence of trisomy 21 was found in one (0.37%) of 269 women aged 34 or under and in none of the 85 women aged 35 or over. No recurrence of trisomy 18 (0/50), trisomy 13 (0/12), or 45X (0/7) was found.

Among Group II singleton fetuses ($n = 394$), we analyzed associations between chromosome abnormalities and prenatal ultrasound findings. Table 3 summarizes the frequency of chromosome abnormalities according to the presence of fetal abnormality, abnormality of amniotic fluid volume (oligohydraminios/polyhydraminios), and fetal growth restriction. A frequency of chromosome abnormality ($n = 14$, 41.2%) in 34 fetuses with fetal abnormality plus polyhydramnios was significantly ($P < 0.01$) higher than ($n = 49$, 19.1%) in 257 fetuses with fetal abnormality plus normal amniotic fluid volume.

Percentages of abnormal ultrasound findings detected in Group II singleton fetuses with trisomy 21, trisomy 18, and 45X are summarized in Table 4. We used univariate analyses to find associations

Table 3 Frequency of chromosome abnormalities according to fetal abnormality, abnormality of amniotic fluid volume and fetal growth restriction (FGR)

	With FGR / Without FGR		
	Normal amniotic fluid volume	Oligohydramnios	Polyhydramnios
Fetal abnormality	25.0% (2/8) / 18.9% (47/249)	100% (1/1) / 0% (0/5)	80.0% (4/5) / 34.5% (10/29)
No fetal abnormality	10.7% (3/28) / NA	0% (0/5) / 0% (0/2)	0% (0/4) / 13.8% (8/58)

NA, not applicable.

Table 4 Percentage of abnormal ultrasound findings detected in Group II singleton pregnancies with trisomy 21, trisomy 18, and 45X fetuses

Abnormal ultrasound findings	Trisomy 21 ($n = 24$)	Trisomy 18 ($n = 21$)	45X ($n = 8$)
Polyhydramnios	16.7	57.1	0
Fetal growth restriction	0	28.6	0
Hydrops fetalis	8.3	4.8	50.0
Pleural effusion	8.3	0	25.0
Ascites	25.0	0	12.5
Abnormality of central nervous system	4.2	23.8	0
Microcephaly	0	4.8	0
Ventriculomegaly	0	4.8	0
Choroid plexus cyst	4.2	4.8	0
Hypoplastic cerebellum	0	9.5	0
Cystic hygroma	33.3	4.8	75.0
Nuchal translucency	12.5	4.8	0
Cardiovascular abnormality	8.3	14.3	12.5
Hypoplastic lung	4.2	0	0
Diaphragmatic hernia	0	4.8	0
Obstruction of digestive tract	8.3	19.0	0
Omphalocele	0	19.0	0
Urinary tract abnormality	0	4.8	0
Abnormal extremity	12.5	9.6	0
Short limb	12.5	0	0
Bent wrist	0	4.8	0
Overlapping finger	0	4.8	0

Table 5 Results of univariate analyses of abnormal ultrasound findings in Group II singleton pregnancies

Abnormal ultrasound findings/advanced maternal age	Cases with chromosome abnormality (%)	Odds ratio (95% CI)	*P*-values
Cystic hygroma	17/35 (48.6)	4.9 (2.4–10.1)	0.001
Abnormal extremity	7/15 (46.7)	4.0 (1.4–11.4)	0.005
Cardiovascular abnormality	6/15 (40.0)	3.0 (1.0–8.7)	0.035
Hydrops fetalis	10/27 (37.0)	2.7 (1.2–6.2)	0.014
Advanced maternal age (≥35 years)	21/65 (32.3)	2.4 (1.3–4.4)	0.003
Polyhydramnios	22/96 (22.9)	1.4 (0.8–2.4)	NS
Oligohydramnios	1/13 (7.7)	0.3 (0.0–2.7)	NS
Fetal growth restriction	10/51 (19.6)	1.0 (0.5–2.2)	NS
Abnormalities of central nervous system	11/61 (18.0)	0.9 (0.5–1.9)	NS
Cleft lip	2/4 (50.0)	4.3 (0.6–31.3)	NS
Nuchal translucency	5/29 (17.2)	0.9 (0.3–2.4)	NS
Fetal arrhythmia	1/9 (11.1)	0.5 (0.1–4.3)	NS
Pleural effusion	4/14 (28.6)	1.7 (0.5–5.7)	NS
Ascites	7/26 (26.9)	1.6 (0.7–4.0)	NS
Diaphragmatic hernia, abdominal wall defect	3/7 (42.9)	3.3 (0.7–15.0)	NS
Omphalocele	8/27 (29.6)	1.9 (0.8–4.5)	NS
Abdominal cyst	1/14 (7.1)	0.3 (0.0–2.5)	NS
Obstruction of digestive tract	6/18 (33.3)	2.2 (0.8–6.1)	NS
Urinary tract abnormality	s1/19 (5.3)	0.2 (0.0–1.7)	NS
Single umbilical artery	2/8 (25.0)	1.4 (0.3–7.2)	NS

NS, not significant.

Table 6 Results of multivariate analyses of abnormal ultrasound findings in Group II singleton pregnancies

Abnormal ultrasound finding	Odds ratio (95% CI)	*P*-value
Cystic hygroma	5.6 (2.7–11.6)	0.0001
Abnormal extremity	5.0 (1.7–14.4)	0.0054
Cardiovascular abnormality	3.3 (1.1–10.1)	0.0372

between chromosome abnormalities and abnormal ultrasound findings or an advanced maternal age (≥35 years old). Cystic hygroma, abnormal extremity, cardiovascular abnormality, hydrops fetalis, and advanced maternal age were significantly related to fetal chromosomal abnormalities (Table 5). By multivariate analyses, abnormal ultrasound findings, including cystic hygroma, abnormal extremity, and cardiovascular abnormality were selected as independent factors associated with fetal chromosomal abnormalities (Table 6).

DISCUSSION

Among 4626 Group I women, advanced maternal age was the most frequent medical indication for CA. One report assessing data of CA from 5484 Japanese women demonstrated that the frequencies of chromosome abnormalities were 1.5% in women aged 35–39 and 3.7% in women aged 40 or over (Yaegashi *et al.* 1998). It was also reported that the frequency of trisomy 21 in women aged 35–39 was approximately 0.42% (Hook 1992). In the current study, we found that frequencies of chromosome abnormalities were 1.4% in women aged 35–39 and 3.5% in women aged 40 or over, and that the frequency of trisomy 21 in women aged 35–39 was 0.2% and increased to 1.5% in women aged 40 or over. Our findings are comparable to the foregoing, suggesting that our study subjects had little or no deviation from the standard population of pregnant women.

We encountered 509 Group I women who had a previous child with a chromosome abnormality. One report demonstrated that the frequencies of chromosome abnormalities among women with a previous child with a chromosome abnormality were 1.3% in women aged 34 or under and 1.8% in women aged 35 or over (Stene & Stene 1984). In the current study, these frequencies were found to be 1.6% (6/378) in women aged 34 or under 0.8% (1/131) in women aged 35 or over. Several reports also assessed recurrence frequencies of the same fetal chromosome abnormality. Stene and Stene (1984) demonstrated that the recurrence frequency of trisomy 21 was 0.8%. The recurrence frequency of trisomy 21 was 1.2%, and no there was no recurrence of trisomy 18 or trisomy 13 in the Japanese population (Uehara *et al.* 1999). In the current study, we found that the recurrence frequency of trisomy 21 was only 0.3%, and no recurrence of trisomy 18 or trisomy 13. The recurrence frequency of trisomy 21 in our study was lower than in the above-mentioned two studies.

Among pregnancies established by assisted reproductive techniques, including *in vitro* fertilization with embryo transfer and ovulation induction, a false-positive rate in maternal serum screening for 21 trisomy increases (Barkai *et al.* 1996; Frishman *et al.* 1997). In the current study, the frequency of chromosome abnor-

malities among women who underwent assisted reproductive techniques was only 1.0% (2/200), but 21 trisomy was not detected. Among these women, an increase in frequency of chromosome abnormalities was not found.

In the current study, as we expected, frequencies of chromosome abnormalities, trisomy 21, trisomy 18, trisomy 13, 45X, deletion or duplication in Group II were significantly higher than in Group I. Among Group II singleton fetuses, we analyzed associations between chromosome abnormality and prenatal ultrasound findings. When polyhydramnios or polyhydramnios together with fetal growth restriction was prenatally detected, the frequencies of chromosome abnormality were found to be 23.4% (22/96) and 44.4% (4/9), respectively. One report presented 22.2% chromosome abnormalities among women with polyhydramnios (Zahn *et al.* 1993). Another report presented 38.5% chromosome abnormalities among women with polyhydramnios together with fetal growth restriction (Sickler *et al.* 1997). These frequencies in Western countries are comparable to our results in the Japanese population. In the current study, among fetuses with abnormality, a frequency of chromosome abnormality in fetuses with polyhydramnios (41.2%) was significantly higher than in fetuses with normal amniotic fluid volume (19.1%). However, polyhydramnios was not selected as a significant factor by univariate analyses.

It has been reported that prenatal ultrasound examinations detected 100% of trisomy 13 fetuses and 77% of trisomy 18 fetuses (Benacerraf *et al.* 1987). Trisomy 21 was related to the presence of hydrops fetalis, cystic hygroma, congenital heart disease, short limbs, and duodenal atresia (Hill *et al.* 1989; Wilson *et al.* 1992.) Ott and Taysi (2001) demonstrated that, by a multivariate analysis, abnormalities of the central nervous system, heart, face and neck; nuchal translucency, hyperechogenic bowel, abnormal extremity, and abnormal biparietal diameter-to-femur length ratio were related to chromosome abnormality of fetuses. In the current study, abnormal ultrasound findings, including cystic hygroma, abnormal extremity, cardiovascular abnormality and hydrops fetalis were significantly related to fetal chromosome abnormality by univariate analyses. By multivariate analyses, we found that cystic hygroma, abnormal extremity, and cardiovascular abnormality were selected as independent factors significantly associated with fetal chromosomal abnormalities. When these ultrasound findings are prenatally detected, obstetricians may consider CA. Prior to CA, evidence-based genetic counseling should be performed. We believe that information revealed in this study constitutes a beneficial reference for discreet genetic counseling.

Risk estimation according to a combination of ultrasound findings, including specific fetal abnormality, abnormality of amniotic fluid volume and fetal growth restriction seemed to be clinically more crucial. Our multiple regression analysis was not able to determine such a combination. The scale of subjects in this study was not small. However, we collected and assessed data accumulated over a period of 24 years. Many circumstances, such as medical information, skill of obstetricians, ultrasound devices and screening systems have changed during that period. These biases could not be excluded in the present study. In the future, prospective studies considering confounding factors are necessary to confirm our results.

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A High Dose of Intravenous Immunoglobulin Increases CD94 Expression on Natural Killer Cells in Women with Recurrent Spontaneous Abortion

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Problem
A high dose of intravenous immunoglobulin (HIVIg) therapy is effective in various diseases such as autoimmune diseases, and also is expected to have efficacy in recurrent spontaneous abortion (RSA). The aim of this study was to understand immunological mechanisms of this therapy.

Method of study
By flowcytometric analyses, we examined phenotypic changes of a variety of immunological cells including natural killer (NK) cells, cytotoxic T cells, regulatory T cells and macrophages in peripheral blood of RSA women with HIVIg therapy ($n = 8$).

Results
Expression percentages of inhibitory CD94 on NK cells significantly ($P = 0.01$) increased after the therapy (58.8 ± 21.4% versus 71.0 ± 17.6%).

Conclusion
Mechanisms of possible efficacy of HIVIg therapy for RSA may include enhancement of CD94 expression and subsequent suppression of NK cell cytotoxicity.

Introduction

A high dose of intravenous immunoglobulin (HIVIg) therapy is effective and widely used in various diseases including idiopathic thrombocytopenic purpura, Guillain–Barré syndrome, Kawasaki's disease, and myasthenia gravis.[1–4] To assess the efficacy of this therapeutic option in women with recurrent spontaneous abortion (RSA) of unexplained etiology, randomized, double-blind, and placebo-controlled trials of a medium dose of intravenous immunoglobulin (IVIg) therapy, in which 20–40 g of immunoglobulin is infused weekly or every 2–4 weeks during early pregnancy, have been performed.[5–10] Conclusions drawn from these IVIg trials are controversial. However, recent reports of meta-analysis[11] and systematic review[12] concerning efficacy of IVIg therapy suggest that a medium dose of IVIg are effective among women with secondary RSA.

On the other hand, our group tried HIVIg therapy for RSA women, in which 100 g of immunoglobulin was infused intravenously over the course of 5 days during early pregnancy. A high live birth rate was

observed among these women who had a history of four or more spontaneous abortions and underwent HIVIg therapy.[13,14] Additionally, using a mouse model of immunological reproductive failure, a recent study demonstrated that intraperitonial injection of a high dose of immunoglobulin restored the fecundity.[15]

We previously reported that HIVIg therapy reduced natural killer (NK) cell activity[14] and Th1/Th2 balance[16] in the blood of RSA women. However, immunological mechanisms of possible efficacy of HIVIg therapy for RSA have not been fully elucidated. In this study, to understand these immunological mechanisms, using flowcytometric analyses we examined phenotypic changes of a variety of immunological cells including NK cells, cytotoxic T cells (CTLs), regulatory T cells (Tregs) and macrophages in peripheral blood of RSA women with HIVIg therapy.

Subjects and methods

Patient Characteristics

Study subjects consisted of eight consecutively seen Japanese patients (30–41 years old), who had a history of four or more (mean 4.9, range 4–7) consecutive abortions, in the Hokkaido University Hospital. The previous abortions of all the eight patients occurred <10 weeks of gestation. Only one patient experienced a full-term normal delivery followed by five consecutive abortions. All the patients had received therapies of luteal hormone, herbal medicine, steroid hormone, low dose aspirin and/or heparin during their previous pregnancies. They underwent examinations of ultrasound, hysterosalpingography, endometrial biopsy and conventional blood analyses for RSA screening, and were diagnosed as having RSA of unexplained etiology. The conventional blood analyses included chromosome karyotypes of couple; measurements of progesterone in mid-luteal phase, prolactin, thyroid, liver, kidney functions, haemostatic coagulation factors such as d-dimer, factor XII, protein C, protein S; and autoimmune factors such as antinuclear antibody, complements, anticardiolipin, beta 2-glycoprotein I-dependent anticardiolipin antibodies and lupus anticoagulant.

They underwent HIVIg therapy (intact type immunoglobulin 20 g daily in the course of 5 days; a total 100 g) with written informed consent immediately after a gestational sac was detected in a uterus by ultrasound. The peripheral blood samples were obtained prior to commencement of HIVIg (4–5 weeks of gestation) and 1–3 days after completion of HIVIg (5–6 weeks of gestation). The gestational age of all pregnancies was determined from basal body temperature.

Two of the eight pregnancies ended in spontaneous abortion. Of the two abortions, one was with chromosome 10 trisomy, and the other with 18 trisomy. The other six pregnancies ended in full-term normal deliveries.

Flow Cytometric Analysis

The peripheral blood samples were suspended in phosphate buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and 0.1% sodium azide. A lysing solution containing NH4Cl and EDTA was added for 10 min at room temperature to lyse the erythrocytes. Peripheral blood cells were washed twice with PBS and resuspended with 1 mL of PBS before flow cytometric analyses using antibodies as follows. For the NK cell analyses, the cells were stained with Peridinin–Chlorophyll–Protein Complex (PerCP)-conjugated anti-CD3 (SK7) mAb (Becton Dickinson, San Jose, CA, USA), fluorescein isothiocyanate (FITC) conjugated anti-CD3 (HIT3a) mAb (Becton Dickinson), allophycocyanin (APC) -conjugated anti-CD56 (NKH-1) (Beckman Coulter, Inc., Fullerton, CA, USA), anti-CD158a (EB6)- R-phycoerythrin (PE) (Immunotech, Marseille, France) and anti-CD94 (HP-3B1)-PE (Immunotech). Cytotoxic T cells were analyzed with mAb as follows: PerCP-conjugated anti-CD3 (SK7) mAb (Becton Dickinson), anti-CD8 (B9.11)-APC (Immunotech) and anti-CD28 (CD28.2)-PE (Immunotech). For the staining of intracellular perforin, the cells were washed and fixed with fixation buffer (CALTAG Laboratories, Burlingame, CA, USA), and then washed with permeabilization buffer (CALTAG). These fixed and permeabilized cells were stained with mouse anti-human perforin (dG9) (Becton Dickinson) with RPE-Cy5-conjugated F(ab)2 fragment of rabbit anti-mouse immunoglobulins (DAKO Cytomation, Glostrup, Denmark), and then stained with anti-CD3 (SK7)-FITC (Becton Dickinson), anti-CD56 (NKH-1)-PE (Beckman Coulter) and anti-CD8 (B9.11)-APC (Immunotech). Tregs were analyzed with mAb as follows: PerCP-conjugated anti-CD4 (SK3) mAb, anti-CD25 (2A3)-APC (Becton Dickinson), anti-FOXP3 (PCH101)-FITC (eBioscience, San Diego, CA, USA), anti-CD28 (CD28.2)-FITC (Im-

munotech), anti-CD152 (BN13)-PE (Immunotech) and anti-CCR4 (1G1)-PE (Becton Dickinson). For the macrophages analyses, the cells were stained with anti-CD163 (GHI/61)-PE (BD Pharmingen, San Diego, CA, USA), FITC-conjugated anti-CD68 (KIM7) mAb (CALTAG Laboratories, Burlingame CA, USA), anti-CD80 (MAB104)-PE (Immunotech), anti-CD86 (FUN-1)- R-phycoerythrin:Cyanine-5.18 (PE-Cy5) (BD Pharmingen), anti-HLA-DR (L243)-PerCP (Becton Dickinson), anti-CD206 (19.2)-APC (BD Pharmingen), mouse anti-MMP-9 (56-2A4)-purified, mouse anti-PPAR-γ (E-8)-purified (BD Pharmingen), goat anti-mouse IgG (H+L)-PE (Beckman Coulter), anti-CD36 (NL07)-APC (BD Pharmingen) and anti-CCL22 (57203)-PE (R&D Systems Inc., Minneapolis, MN, USA).

Three and four color flow cytometric analyses were carried out using a FACS Calibur flow cytometer (Becton Dickinson) and CellQuest Software (Becton Dickinson).

Statistical Analysis

The paired *t*-test was used to analyze the results. $P < 0.05$ was considered as statistically significant.

Results

The gating and frequencies of CD94+ cells in CD3−CD56+ NK cells are shown in Fig. 1.

Table I shows changes in specific cell percentages before and after HIVIg therapy. Percentages of natural killer T (NKT) cells detected as CD3+CD56+ increased after HIVIg therapy, but without a statistical significance ($P = 0.08$). When we examined percentages of NKT cells as Va24+Vb11+ cells/CD3+ CD4−CD8− cells, the percentages (mean ± S.D., 0.59 ± 0.65%) did not significantly change after HIVIg therapy (0.41 ± 0.51%). Percentage of NK cells detected as CD3−CD56+ cells, CTLs detected as CD3+CD8+ cells, or Tregs detected as CD4+CD25+ cells did not significantly change after HIVIg therapy. Percentages of macrophages detected as CD68+ cells increased after HIVIg therapy, but without a statistical significance ($P = 0.06$).

Cell percentages of CD3−CD56+ NK cells expressing perforin or CD158a did not change after HIVIg therapy. However, percentages of NK cells expressing CD94 significantly ($P = 0.01$) increased after

Table I Changes in Specific Cell Percentages

	Before HIVIg (%)	After HIVIg (%)	*P*-value
Natural killer T cells/lymphocytes	4.5 ± 2.9	6.0 ± 4.3	0.08
Natural killer cells/lymphocytes	8.7 ± 3.6	9.0 ± 3.0	NS
Cytotoxic T cells/lymphocytes	21.7 ± 7.4	21.7 ± 6.7	NS
Regulatory T cells/lymphocytes	18.3 ± 7.4	17.6 ± 7.3	NS
Macrophages/all cells	4.8 ± 2.2	6.9 ± 1.9	0.06

HIVIg, a high dose of intravenous immunoglobulin therapy; (Mean ± S.D.).
NS, not significant.

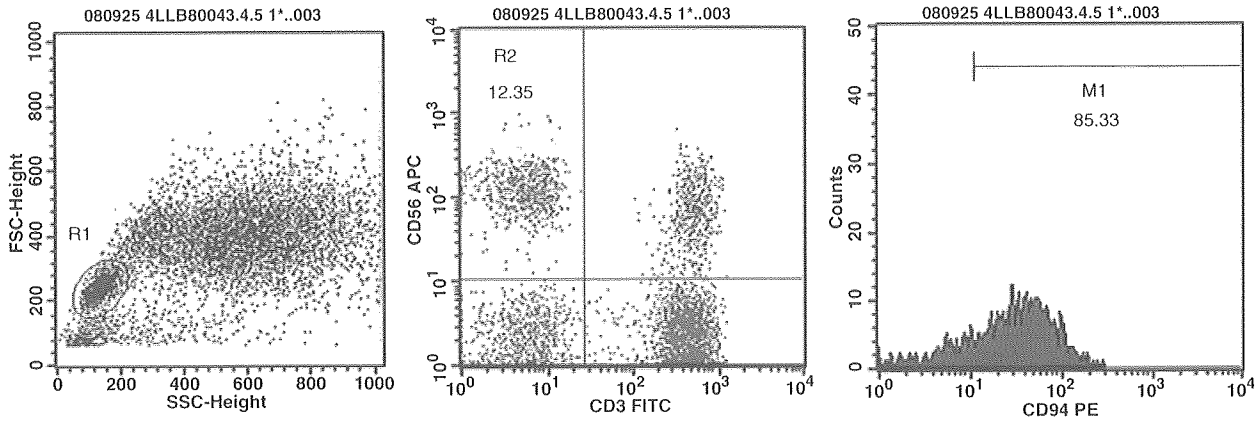

Fig. 1 The gating and frequencies of CD94+ cells in CD3−CD56+ natural killer cells. The peripheral blood was stained with CD3, CD56 and CD94 monoclonal antibodies, as described in Subjects and Methods. A gate (R1) was set on lymphocytes by characteristic forward scatter (FSC) and side scatter (SSC). The analysis gate (R2) was set for CD3−CD56+ lymphocytes and they were further identified by R-phycoerythrin fluorescence conjugating to anti-CD94 monoclonal antibody. CD94+ natural killer cells were detected as a distinct population as marker 1 (M1).

Table II Changes in Percentages of CD3−CD56+ Natural Killer Cells Expressing Specific Molecules

	Before HIVIg (%)	After HIVIg (%)	*P*-value
CD3−CD56+ natural killer cells			
Perforin	89.9 ± 4.7	89.4 ± 4.8	NS
CDI58a	21.7 ± 5.2	22.6 ± 4.6	NS
CD94	58.8 ± 21.4	71.0 ± 17.6	0.01

HIVIg, a high dose of intravenous immunoglobulin therapy (Mean ± S.D.)
NS, not significant.

HIVIg therapy (Table II). When two abortion cases with fetal chromosome abnormality were excluded, percentages of NK cells expressing CD94 more significantly ($n = 6$, $P = 0.003$) increased after the therapy. Fig. 2 shows individual changes in percentages of CD94+ NK cells.

Cell percentages of CD3+CD8+ CTLs expressed perforin in similar ratio before and after HIVIg therapy. Percentages of CD3+CD8+ CTLs expressing CD28 decreased after HIVIg therapy, but with a borderline significance ($P = 0.05$) (Table III).

Cell percentages of CD4+CD25+ Tregs expressing Foxp3, CD28, CD152, or CCR4 did not change after HIVIg (Table IV).

No change was observed in the percentages of CD68+ macrophages expressing CD80, CD86, MMP9, CD206, CD163, HLA-DR, PPAR-γ, CD36, or CCL22 after HIVIg therapy (Table V).

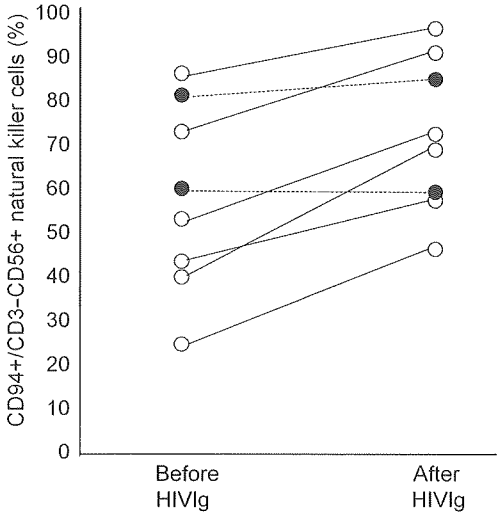

Fig. 2 Changes in percentages of CD94+ natural killer cells; HIVIg, a high dose of intravenous immunoglobulin therapy. Open circles indicate women whose pregnancies ended in live births ($n = 6$); and closed circles indicate women whose pregnancies ended in spontaneous abortion ($n = 2$).

Discussion

The possible pharmacodynamic mechanisms of HIVIg efficacy for clinical use involve interaction of anti-idiotype antibodies with pathologic antibodies, suppression of inflammation, and modification of Fc receptor, T cell, B cell or macrophage functions.[4] We for the first time reported possible efficacy of HIVIg therapy in severe cases of RSA.[13,14] However, immunological mechanisms of HIVIg therapy in RSA have been poorly elucidated.

Concerning NK cell abnormality in spontaneous abortion (SA), it was reported that peripheral NK cell activities were abnormally elevated prior to conception and during early pregnancy in RSA women whose pregnancies were destined to end in SA with normal fetal chromosome karyotype (SANC), but this NK cell accentuation was not detected in RSA women with spontaneous abortion with abnormal fetal chromosome karyotype (SAAC).[17,18] Recently, we found decreased expression of CD94 molecules, which are inhibitory receptors on NK cells, in the decidua of women with sporadic SANC when compared with women with sporadic SAAC or women with induced abortions.[19] These results suggested that the high activity and decrease in inhibitory receptor expression of NK cells might be causally associated with RSA as well as spontaneous abortion.

We previously demonstrated that peripheral NK cell activity was effectively suppressed by HIVIg therapy in RSA women,[14] and demonstrated that expression of inhibitory CD94 was inversely correlated with expression of cytotoxic molecule, perforin on NK cells in the deciduae.[19] In this study, we found no change in peripheral NK cell percentages, but

Table III Changes in Percentages of CD3+ CD5+ Cytotoxic T cells Expressing Specific Molecules

	Before HIVIg (%)	After HIVIg (%)	*P*-value
CD3+CD8+ cytotoxic T cells			
Perforin	24.5 ± 8.4	28.5 ± 11.0	NS
CD28	77.0 ± 5.5	70.9 ± 9.7	0.05

HIVIg, a high dose of intravenous immunoglobulin therapy (mean ± S.D.)
NS, not significant.

Table IV Changes in Percentages of CD4+CD25+ Regulatory T cells Expressing Specific Molecules

	Before HIVIg (%)	After HIVIg (%)	*P*-value
CD4+CD25+ regulatory T cells			
Foxp3	15.6 ± 8.4	15.7 ± 6.2	NS
CD28	99.2 ± 1.3	99.0 ± 1.2	NS
CD152	14.1 ± 12.3	15.5 ± 8.8	NS
CCR4	51.0 ± 7.6	49.9 ± 7.9	NS

HIVIg, a high dose of intravenous immunoglobulin therapy; (mean ± S.D.).
NS, not significant.

Table V Changes in percentages of CD68+ macrophages expressing specific molecules

	Before HIVIg (%)	After HIVIg (%)	*P*-value
CD68+ macrophages			
CD80	2.8 ± 2.1	2.3 ± 1.5	NS
CD86	77.2 ± 15.3	74.5 ± 15.1	NS
MMP9	24.4 ± 40.2	17.5 ± 30.0	NS
CD206	0.9 ± 1.1	1.1 ± 1.7	NS
CD163	1.6 ± 2.2	1.8 ± 1.9	NS
HLA-DR	77.6 ± 9.4	75.6 ± 13.4	NS
PPAR-γ	9.7 ± 9.3	7.3 ± 6.8	NS
CD36	95.4 ± 5.7	95.2 ± 3.3	NS
CCL22	3.8 ± 4.2	3.8 ± 2.3	NS

HIVIg, a high dose of intravenous immunoglobulin therapy: (mean ± SD).
NS, not significant.

found that expression of inhibitory CD94 on NK cells increased after HIVIg therapy. The expression of inhibitory CD94 was more significantly enhanced by the therapy in six women with live births. Conversely, CD94 expression showed no or little increase in two women with SAAC. The increase in CD94 expression on NK cells resulting from the therapy might be related to desirable fetal prognosis and pharmacodynamic mechanisms of HIVIg efficacy. However, in this study, we experienced no SANC. If we could understand changes in CD94 expression among enough number of women with SANC and SAAC, we would discuss whether an increase in CD94 expression predicted fetal prognosis, or was merely a reflection of HIVIg therapy. It is thought that the activity/cytotoxicity of NK cells results from expression balances of various activating receptors and inhibitory receptors on NK cells.[20–22] Inhibitory

receptor complexes (CD94-NKG2A) belong to the C-type lectin superfamily and contain ITIM sequences in their cytoplasmic tails.[22] HIVIg therapy increases the expression of the inhibitory CD94 receptors and subsequently suppresses NK cell activity/cytotoxicity in the body of RSA women. This is a hypothetical mechanism of possible efficacy of HIVIg therapy for RSA.

It is known that the number of NKT cells increases in the deciduae during early pregnancy;[23] and NKT cells may control the Th cell function at the materno-fetal interface through the production of IFN-γ and IL-4.[24] One report demonstrated that increased number of NKT cells in women with RSA or implantation failure was ameliorated by IVIg therapy, leading to successful pregnancy outcome.[25] In this study, however, NKT cell percentages were not significantly changed by HIVIg therapy.

CD3+CD8+ CTLs may play a role in RSA etiologies. We reported increased expression of perforin on CTLs in the deciduae of women with sporadic SANC when compared with women with sporadic SAAC or women with induced abortions. The expression of perforin on CTLs was inversely correlated with expression of inhibitory CD94 on NK cells in the deciduae.[19] In this study, we measured expression of perforin and CD28 on CTLs. CD28 is a ligand of CD80 and CD86 on antigen presenting cells, and can transduce an activating signal. CD28 expression on CTLs decreased after HIVIg therapy, but with a borderline significance ($P = 0.05$). HIVIg therapy may decrease CD28 expression and subsequently suppress activating signal transduction on CTLs in RSA women. This hypothesis should be further clarified. We measured expression of Treg associated molecules including FOXP3, CD28, CD152 (CTLA-4), and CCR4 in CD4+CD25+ T cells. HIVIg therapy did not cause significant changes in expression percentages of these molecules.

Using a mouse model of immunological reproductive failure, we recently demonstrated that intraperitonial injection of a high dose of immunoglobulin restored the fecundity.[15] Additionally, we found that spleen cells adoptively transferred from immunoglobulin injected donors to recipient mice of reproductive failure restored the fecundity. CD11b+ macrophages transferred from donor mice accumulated selectively in the placentae of recipient mice.[15] Therefore, we expected that macrophages might play a key role in mechanisms of HIVIg efficacy in RSA women. In this study, percentages of macrophages increased after HIVIg therapy, but without statistical significance ($P = 0.06$). Expression of macrophage associated molecules including CD80, CD86, MMP9, CD206, CD163, HLA-DR, PPAR-γ, CD36, or CCL22 in the peripheral blood was not changed by HIVIg therapy. Further investigations are needed.

In this study, we experienced desirable pregnancy outcome, i.e., live births after HIVIg therapy in six patients who had a history of four or more abortions of unexplained etiology. We performed the therapy only once during their early pregnancies; and no additional infusion of immunoglobulin was needed. We believe that HIVIg as immune modifier is effective when this therapy is performed during early pregnancy. Severe RSA cases may have immunologic abnormality as its etiology that can be corrected by HIVIg in early pregnancy.

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Anti-β2 glycoprotein-I antibody increases the risk of pregnancy-induced hypertension: a case-controlled study

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ABSTRACT

The aim of this study was to evaluate whether anti-β2 glycoprotein-I antibody (anti-β2GPI) of the IgG or IgM classes is associated with the development of pregnancy-induced hypertension (PIH) or preeclampsia in the Japanese population. In a case-controlled cohort study, peripheral blood was obtained at 8–14 weeks of gestation from a consecutive series of 1155 women. The case group comprised 36 patients who later developed PIH during the pregnancy. Of the 36 PIH patients, 13 had severe PIH, 18 had preeclampsia and 11 had severe preeclampsia. One hundred and eleven age- and parity-matched women whose pregnancies ended in normal delivery without obstetric complications were selected as controls. We found that a titer of anti-β2GPI IgG ≥ 1.0 U/ml was a risk factor for severe PIH ($P = 0.023$, OR 5.7 95%CI 1.4–22.8). In addition, titers of anti-β2GPI IgM ≥ 1.2 U/ml was found to be a risk factor for PIH ($P = 0.001$, OR 8.8 95%CI 1.6–47.5). In women positive for anti-β2GPI but negative for lupus anticoagulant, anti-cardiolipin, phosphatidylserine-dependent anti-prothrombin, or kininogen-dependent anti-phosphatidylethanolamine antibodies, the presence of anti-β2GPI was not a significant risk factor for development of PIH or preeclampsia. In conclusion, the presence of anti-β2GPI antibody represents a risk factor for developing PIH and severe PIH. This finding supports the utility of anti-β2GPI determination as one of the laboratory criteria for anti-phospholipid syndrome classification. The usefulness of anti-β2GPI measurement among women without other anti-phospholipid antibodies requires further study.

1. Introduction

Anti-phospholipid antibodies (aPLs) are a heterogeneous group of autoantibodies directed against phospholipid-binding proteins. Anti-phospholipid syndrome (APS) refers to the association between aPLs and thrombosis or pregnancy morbidity. Criteria used widely as a consensus definition for APS were established by the Eighth International Symposium on Anti-phospholipid Antibodies Syndrome in Sapporo (Wilson et al., 1999). Obstetric complications included in this APS definition are recurrent pregnancy loss, unexplained fetal death, severe preeclampsia, intrauterine growth restriction, and premature delivery. Two types of aPLs were originally included in the laboratory criteria: anti-cardiolipin antibody (aCL) (either IgG or IgM), and lupus anticoagulant. In 2006, amendments to the Sapporo APS criteria were proposed at a workshop preceding the Eleventh International Congress on aPLs. Consequently, anti-β2 glycoprotein-I antibody (anti-β2GPI) of the IgG or IgM class was included as a

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laboratory criteria for the classification of definite APS (Miyakis et al., 2006).

Pregnancy-induced hypertension (PIH) and preeclampsia are amongst the major causes of mortality and morbidity during pregnancy and childbirth. The pathogenesis is multifactorial. PIH and preeclampsia can lead to multiple organ failure involving the cardiovascular and central nervous systems, the liver, and kidneys as well as cause coagulation breakdown. However, the association between aPLs and the risk of PIH and preeclampsia still remains controversial. Recently, we prospectively assessed aPLs including lupus anticoagulant, aCL, phosphatidylserine-dependent anti-prothrombin antibody, and kininogen-dependent anti-phosphatidylethanolamine antibody (aPE) in 1155 consecutive women during early pregnancy. We demonstrated that a positive test for aCL IgG or aPE IgG was associated with developing PIH during the third trimester in the SAPPORO study (Yamada et al., 2009). However, anti-β2GPI was not assessed in this study.

The present study was performed as a case-controlled study in a cohort of the SAPPORO study population to evaluate whether anti-β2GPI was associated with PIH or preeclampsia.

2. Materials and methods

2.1. Subjects

A previous study, designated Sapporo Multiple Antiphospholipid Testing for the Prediction of Obstetric Outcome study (the SAPPORO study) was performed in the city of Sapporo, Japan, and conducted with informed consent from all of the subjects (Yamada et al., 2009). The study was approved by the institutional ethics board of Hokkaido University Graduate School of Medicine. Peripheral blood was obtained at 8–14 weeks of gestation from a consecutive series of 1155 women with living fetuses who visited the Hokkaido University Hospital or an affiliate hospital. The sera were collected and stored at −80 °C.

The present study was performed as a case-controlled study in a cohort from the SAPPORO study population, and comprised 36 cases that developed PIH during pregnancy. The 36 PIH cases (age range 22–41, mean ± SD 32.1 ± 4.6 years old, 25 (69%) nulliparity) included 13 severe PIH, 18 preeclampsia and 11 severe preeclampsia cases. A group of 111 age and parity-matched women whose pregnancies ended in normal delivery without obstetric complications were randomly selected as controls (age range 22–41, mean ± SD 32.1 ± 4.6 years old, 76 (69%) nulliparity). This random selection was performed by one scientist (I.F.) who is not a medical doctor and did not have the knowledge of results of other aPLs.

In this study, we used PIH and preeclampsia criteria defined by the Japan Society of Obstetrics and Gynecology. PIH was defined as hypertension (systolic blood pressure > 140 mm Hg or diastolic blood pressure > 90 mm Hg) detected after 20 weeks gestation. Severe PIH was defined when at least one of the following criteria was met: (1) blood pressure ≥ 160/110 mm Hg after 20 weeks gestation, regardless of the complication of proteinuria, or (2) blood pressure ≥140/90 mm Hg after 20 weeks gestation complicated by proteinuria ≥2.0 g/day. Preeclampsia was defined as hypertension (≥140/90 mm Hg) and proteinuria (≥300 mg/day) detected after 20 weeks gestation. Severe preeclampsia was defined when at least one of the following criteria was met: (1) blood pressure ≥160/110 mm Hg after 20 weeks gestation complicated by proteinuria ≥300 mg/day, or (2) blood pressure ≥140/90 mm Hg after 20 weeks gestation complicated by proteinuria ≥2.0 g/day. Blood pressures were measured repeatedly.

2.2. Anti-phospholipid antibody measurement

Anti-β2GPI antibodies were measured by ELISA in the stored sera using a protocol reported previously (Amengual et al., 1996). Purified human β2GPI was purchased from Yamasa Corp. Tokyo, Japan. Irradiated microtitre plates (Maxisorp, Nunc, Denmark) were coated with 4 μg/ml of purified β2GPI in phosphate-buffered saline (PBS) at 4 °C and washed twice with PBS. To avoid non-specific binding of proteins, wells were blocked with 150 μl of 3% gelatin (BDH Chemicals Ltd., Poole). After three washes with PBS containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO, USA) (PBS–Tween), 50 μl of serum sample diluted 1:50 with PBS containing 1% bovine serum albumin (Sigma) (PBS–1% BSA) was added to duplicate wells. Plates were incubated for 1 h at room temperature and washed three times with PBS–Tween. Fifty microlitres per well of the appropriate dilution of alkaline phosphatase-conjugated goat anti-human IgG and IgM (Sigma) in PBS–1%BSA was added. After 1 h of incubation at room temperature and after four washes in PBS–Tween, 100 μl/well of 1 mg/ml p-nitrophenylphosphate disodium (Sigma) in 1 M diethanolamine buffer (pH 9.8) was added. Following colour development, optical density at 405 nm was measured by a Multiskan ascent plate reader (Thermo electron corporation, Waltham MA, USA).

One of the serum samples that had showed high binding to β2GPI coated onto the irradiated plates was used as a positive control. Normal ranges of anti-β2GPI IgG (<2.2 U/ml) and IgM (<6.0 U/ml) with cut-off values at the 99th percentile were established in preliminary experiments using non-pregnant 132 healthy controls. Cut-off values of anti-β2GPI IgG (normal < 1.0 U/ml) and anti-β2GPI IgM (normal < 1.2 U/ml) were established as the most appropriate values dividing the subjects in this study. To define the intra-assay precision for anti-β2GPI IgG and IgM, 3 patient samples with high, medium and low titers of anti-β2GPI IgG or IgM were replicated 12 times on the same 96-well ELISA plate, and coefficients of variation were calculated from the optical density results.

2.3. Statistical analysis

Statistical differences were analyzed by the chi-square test (df = 1). Fisher's exact test was used when the number of observations was ≤5. A $P < 0.05$ was considered statistically significant. Odds ratios (OR) and 95% confidence intervals (95%CI) were calculated to evaluate the association between anti-β2GPI and PIH or preeclampsia. All statistical analyses were conducted with a statistical anal-

Table 1
IgG anti-β2 glycoprotein-I as a risk factor for pregnancy-induced hypertension and preeclampsia with a cut-off value or 2.2 U/ml.

Outcome	Positive frequency of IgG anti-β2GPI						*P* value	Odds ratio	95% confidence intervals
	Women with positive other aPL[a]		Women with negative other aPL[a]		Total				
Normal (controls)	1/12	8.3%	5/99	5.1%	6/111	5.4%			
PIH	2/8	25.0%	2/28	7.1%	4/36	11.1%	0.26	2.2	0.6–8.3
Severe PIH	2/5	40.0%	1/8	12.5%	3/13	23.1%	0.053	5.3	1.1–24.3
Preeclampsia	1/4	25.0%	1/14	7.1%	2/18	11.1%	0.31	2.2	0.4–11.8
Severe preeclampsia	1/3	33.3%	1/8	12.5%	2/11	18.2%	0.15	3.9	0.7–22.1

PIH, pregnancy-induced hypertension; anti-β2GPI, anti-β2 glycoprotein-I antibody; aPL, anti-phospholipid antibody.
[a] Other aPLs include IgG/IgM anti-cardiolipin antibody, lupus anticoagulant, IgG/IgM phosphatidylserine-dependent anti-prothrombin antibody, and IgG kininogen-dependent anti-phosphatidylethanolamine.

ysis system package (SAS version 9.1, SAS Institute Japan Ltd., Tokyo, Japan).

3. Results

The numbers of women with a positive anti-β2GPI IgG test of ≥2.2 U/ml (or ≥1.0 U/ml) were as follows: 6 (8) in 111 controls, 4 (6) in 36 PIH, 3 (4) in 13 severe PIH, 2 (4) in 18 preeclampsia, and 2 (3) in 11 severe preeclampsia. We found no women tested positive for ≥6.0 U/ml of anti-β2GPI IgM. The numbers of women with a positive anti-β2GPI IgM test of ≥1.2 U/ml were as follows: 2 in controls, 5 in PIH, 2 in severe PIH, 2 in preeclampsia, and 1 in severe preeclampsia. The anti-β2GPI intra-assay coefficients of variation for the high, medium and low positive samples were 4.4%, 4.1% and 8%, respectively for the IgG assay, and 4.6%, 5.4% and 8.8% for the IgM assay, representing good assay precision.

The results of statistical analyses are shown in Tables 1–3. A titer ≥1.0 U/ml of anti-β2GPI IgG was found to be a significant risk factor for severe PIH (P = 0.023, OR 5.7 95%CI 1.4–22.8) (Table 2). A titer ≥1.2 U/ml of anti-β2GPI IgM was a significant risk factor for PIH (P = 0.001, OR 8.8 95%CI 1.6–47.5) (Table 3). We also found a possible association between titers ≥1.0 U/ml of anti-β2GPI IgG and severe preeclampsia (P = 0.061, OR 4.8 95%CI 1.1–21.8) (Table 2), between titers ≥2.2 U/ml of anti-β2GPI IgG and severe PIH (P = 0.053, OR 5.3 95%CI 1.1–24.3) (Table 1), and between titers ≥1.2 U/ml of anti-β2GPI IgM and severe PIH (P = 0.054, OR 9.9 95%CI 1.3–77.4) (Table 3). However, these associations did not reach statistical significance.

Of the 6 PIH women with titers ≥1.0 U/ml of anti-β2GPI IgG, one had aPE, another had aCL plus lupus anticoagulant, and the other 4 women had neither lupus anticoagulant, aCL, phosphatidylserine-dependent anti-prothrombin antibody, nor aPE. Among the 30 PIH women with a negative test of anti-β2GPI IgG, five had aPE IgG, one had aCL plus phosphatidylserine-dependent anti-prothrombin antibody, and the other 24 women had no aPLs. Of the 8 control women with titers ≥1.0 U/ml of anti-β2GPI IgG, two had phosphatidylserine-dependent anti-prothrombin antibody, and the other 6 women had no aPLs. Of the 103 control women testing negative for anti-β2GPI IgG, eight had aPE IgG, two had phosphatidylserine-dependent anti-prothrombin antibody, one had aCL, one had lupus anticoagulant, and the other 91 women had no aPLs. These aPL characterisics were quoted from known data in the SAPPORO study (Yamada et al., 2009).

Similarly, of the 5 PIH women with titers ≥1.2 U/ml of anti-β2GPI IgM, one had aCL plus lupus anticoagulant, another had aCL plus phosphatidylserine-dependent anti-prothrombin antibody and the other 3 women had neither lupus anticoagulant, aCL, phosphatidylserine-dependent anti-prothrombin antibody, nor aPE. Among the 31 PIH women testing negative for anti-β2GPI IgM, six had aPE IgG, and the other 25 women had no aPLs. Of the 2 control women with titers ≥1.2 U/ml of anti-β2GPI

Table 2
IgG anti-β2 glycoprotein-I as a risk factor for pregnancy-induced hypertension and preeclampsia with a cut-off value of 1.0 U/ml.

Outcome	Positive frequency of IgG anti-β2GPI						*P* value	Odds ratio	95% confidence intervals
	Women with positive other aPL[a]		Women with negative other aPL[a]		Total				
Normal (controls)	2/12	16.7%	6/99	6.1%☆	8/111	7.2%*			
PIH	2/8	25.0%	4/28	14.3%☆	6/36	16.7%	0.065	2.6	0.8–8.8
Severe PIH	2/5	40.0%	2/8	25.0%☆	4/13	30.8%*	0.023	5.7	1.4–22.8
Preeclampsia	1/4	25.0%	3/14	21.5%☆	4/18	22.2%	0.065	3.2	0.9–11.9
Severe preeclampsia	1/3	33.3%	2/8	25.0%☆	3/11	27.3%	0.061	4.8	1.1–21.8

PIH, pregnancy-induced hypertension; anti-β2GPI, anti-β2 glycoprotein-I antibody; aPL, antiphospholipd antibody.
[a] Other aPLs include IgG/IgM anti-cardiolipin antibody, lupus anticoagulant, IgG/IgM phosphatidylserine-dependent anti-prothrombin antibody, and IgG kininogen-dependent anti-phosphatidylethanolamine.
* Statistically significant (P = 0.023).
☆ Not significant (P > 0.05).

Table 3
IgM anti-β2 glycoprotein-I as a risk factor for pregnancy-induced hypertension and preeclampsia with a cut-off value of 1.2 U/ml.

Outcome	Positive frequency of IgM anti-β2GPI						*P* value	Odds ratio	95% confidence intervals
	Women with positive other aPL[a]		Women with negative other aPL[a]		Total				
Normal (controls)	0/12	0%	2/99	2.0%☆	2/111	1.8%*			
PIH	2/8	25.0%	3/28	10.7%☆	5/36	13.9%*	0.001	8.8	1.6–47.5
Severe PIH	1/5	20.0%	1/8	12.5%☆	2/13	15.4%	0.054	9.9	1.3–77.4
Preeclampsia	0/4	0%	2/14	14.3%☆	2/18	11.1%	0.093	6.8	0.9–51.8
Severe preeclampsia	0/3	0%	1/8	12.5%☆	1/11	9.1%	0.249	5.5	0.5–65.5

PIH, pregnancy-induced hypertension; anti-β2GPI, anti-β2 glycoprotein-I antibody; aPL, anti-phospholipid antibody.
[a] Other aPLs include IgG/IgM anti-cardiolipin antibody, lupus anticoagulant, IgG/IgM phosphatidylserine-dependent anti-prothrombin antibody, and IgG kininogen-dependent anti-phosphatidylethanolamine.
* Statistically significant ($P = 0.001$).
☆ Not significant ($P > 0.05$).

IgM, none had aPLs. Of the 109 control women testing negative for anti-β2GPI IgM, eight had aPE IgG, two had phosphatidylserine-dependent anti-prothrombin antibody, one had aCL, one had lupus anticoagulant, and the other 97 women had no aPLs.

Among women negative for other aPL, the incidence of positivity for anti-β2GPI IgG (≥2.2 U/ml) in women with PIH ($P = 0.96$, OR 1.5 95%CI 0.3–7.9), severe PIH ($P = 0.93$, OR 2.7 95%CI 0.3–26.3), preeclampsia ($P = 0.75$, OR 1.4 95%CI 0.16–13.4) or severe preeclampsia ($P = 0.93$, OR 2.7 95%CI 0.3–26.3), was not significantly different from those in controls (Table 1). Similarly, among women negative for other aPL, the incidence of positivity for anti-β2GPI IgG (≥1.0 U/ml) in women with PIH ($P = 0.30$, OR 2.6 95%CI 0.7–9.9), severe PIH ($P = 0.21$, OR 5.2 95%CI 0.9–31.3), preeclampsia ($P = 0.14$, OR 4.2 95%CI 0.9–19.3) or severe preeclampsia ($P = 0.21$, OR 5.2 95%CI 0.9–31.3), was not significantly higher than in controls (Table 2). Among women negative for other aPL, the incidence of positivity for anti-β2GPI IgM (≥1.2 U/ml) in groups with PIH ($P = 0.12$, OR 5.8 95%CI 0.9–36.7), severe PIH ($P = 0.54$, OR 6.9 95%CI 0.6–86.1), preeclampsia ($P = 0.12$, OR 8.1 95%CI 1.0–62.7) or severe preeclampsia ($P = 0.54$, OR 6.9 95%CI 0.6–86.1) was not significantly higher than in controls (Table 3).

4. Discussion

It has been reported that anti-β2GPI is associated with increased risk of recurrent spontaneous abortion and pregnancy loss among women with aPLs such as lupus anticoagulant (Falcón et al., 1997; Forastiero et al., 1997; Lee et al., 1999; Sailer et al., 2006), and among women without lupus anticoagulant (Stern et al., 1998). Conversely, other studies did not find an association (Ailus et al., 1996; Arnold et al., 2001). Previous prospective studies assessing associations between anti-β2GPI and PIH or preeclampsia also found conflicting results. One report noted that preeclampsia and eclampsia were related to the presence of anti-β2GPI in the maternal blood (Faden et al., 1997). However, other studies reported no association between anti-β2GPI and PIH (Lynch et al., 1999), or preeclampsia and HELLP syndrome (Lee et al., 2003). In the present study, we demonstrated that a positive test of anti-β2GPI IgG in early pregnancy was a significant risk factor for later developing severe PIH; and that a positive test of anti-β2GPI IgM was a significant risk factor for later developing PIH. It seems that the rate of positive anti-β2GPI test in a subset of severe cases is higher than those in the total PIH group. Of the PIH women with anti-β2GPI IgG or IgM, only one patient had lupus anticoagulant, suggesting that our study population was little affected by other aPLs. However, our results contrast with the lack of association observed in the abovementioned two cohort studies (Lynch et al., 1999; Lee et al., 2003). Most subjects of the former study included mild, but not severe PIH (Lynch et al., 1999). The latter study showed low frequencies of positive anti-β2GPI IgG test in controls (2%) and severe PIH (2%) using their cut-off values (Lee et al., 2003). The discrepancy may be explained by the difference in populations included in the studies, or related to the definition of the cut-off levels. Anti-β2GPI assays are not universally standardized, which leads to inter-laboratory variation.

There is a large body of evidence for an involvement of anti-β2GPI in hypercoagulation status and thrombosis (Martinuzzo et al., 1995; Amengual et al., 1996; Zanon et al., 1999; Zoghlami-Rintelen et al., 2005; Pengo et al., 2005; de Laat et al., 2004). A multivariate analysis in a multicenter study has demonstrated that anti-β2GPI and aPE, but not lupus anticoagulant or aCL, were significantly associated with thrombosis (Sanmarco et al., 2007). Anti-β2GPI induces activation of endothelial cells, resulting in a proinflammatory state which favours prothrombotic diathesis (D'Ippolito et al., 2007). Recently, a study has demonstrated β2GPI can naturally inhibit von Willebrand factor (VWF)-dependent platelet adhesion and aggregation. Anti-β2GPI of APS patients neutralized the β2GPI–VWF interactions, contributing to hypercoagulation status in these patients (Hulstein et al., 2007). It is likely that the thrombotic insult of anti-β2GPI to placental angiogenesis or the circulation is causally associated with PIH. Additionally, β2GPI binds to trophoblast cells (Di Simone et al., 2007). Antibody binding to β2GPI downregulates trophoblast chorionic gonadotropin synthesis and secretion (Di Simone et al., 2005). Such a direct effect on trophoblast may contribute to inhibition of trophoblast invasiveness and lead to defective placentation (Di Simone et al., 2007), and may be causally associated with PIH.