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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MHR ORIGINAL RESEARCH

Proinflammatory cytokine polymorphisms and the risk of preterm birth and low birthweight in a Japanese population

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ABSTRACT: Pregnancy and parturition involve a complex and poorly understood molecular and biological interplay between mother and fetus. Inflammatory cytokines have been reported to be associated with fetal growth and parturition. The aim of this study was to examine whether common proinflammatory cytokine polymorphisms are associated with preterm birth (PTB), low birthweight or intrauterine growth restriction in a Japanese population. We assessed a consecutive series of 414 women who had singleton deliveries in Sapporo, Japan between 2001 and 2005. Genotyping of *IL1A* −889C/T, +4845G/T (A114S), *IL1B* −511C/T, −31C/T, *IL2* −384T/G and *IL6* −634C/G polymorphisms was determined by an allelic discrimination assay. The risk of PTB significantly increased in women carrying the *IL1A* −889T allele (CC genotype [reference]; CT genotype, odds ratios (OR): 2.5; 95% confidence intervals (95% CI): 1.4–4.8; CT+TT genotypes [dominant genotype model], OR: 2.5, 95% CI: 1.3–4.6). Similarly, the risk of PTB significantly increased in women carrying the *IL1A* +4845T allele (GG genotype [reference]; GT genotype, OR: 2.4, 95% CI: 1.3–4.4; GT+TT genotypes [dominant genotype model], OR: 2.3, 95% CI: 1.2–4.2). The frequency of the *IL1A* TT haplotype in mothers with PTB was significantly higher than in mothers who had a term birth ($P < 0.001$), whereas the frequency of the *IL1A* CG haplotype in mothers who had a PTB was significantly lower ($P < 0.001$). Our findings suggest that the polymorphisms and haplotypes in the *IL1A* gene are associated with PTB in Japanese women.

Key words: cytokines/growth factors / gene mutations / haplotype / preterm birth / low birthweight

Introduction

Preterm birth (PTB), a birth at fewer than 37 weeks of gestation, is a major public health concern because of its high prevalence, associated mortality and morbidity, and the expense of both hospitalization and risk of long-term disability (Goldenberg *et al.*, 2000; Crider *et al.*, 2005). PTB occurs in 5–10% of births and is associated with 70–80% of neonatal mortality (Goldenberg *et al.*, 2000). Low birthweight (LBW), a birthweight <2500 g, does not always accompany PTB, but is also associated with increased neonatal morbidity and mortality (McCormick, 1985; Henriksen, 1999).

Over the last decade, it has become increasing apparent that the cause of PTB is multi-factorial and involves both genetic and environmental factors (Santtila *et al.*, 1998; Dominici *et al.*, 2002; Genc *et al.*, 2002; Annells *et al.*, 2004; Moore *et al.*, 2004; Engel *et al.*, 2005; Edwards *et al.*, 2006; Pennell *et al.*, 2007). Family, twins and *trans*-generational studies have provided evidence that PTB is heritable in some cases (Porter *et al.*, 1997; Clausson *et al.*, 2000; Treloar *et al.*, 2000; Ward *et al.*, 2005). The genes involved in inflammatory processes and the immune system may be one of the most likely targets in the etiology of PTB (Romero *et al.*, 1989a, b; Dinarello, 1991; Hillier *et al.*, 1993; Greig *et al.*, 1997; Santtila *et al.*, 1998; Dominici *et al.*, 2002; Annells *et al.*, 2004; Moore *et al.*, 2004; Crider *et al.*, 2005; Engel *et al.*, 2005; Edwards *et al.*, 2006).

Although parturition involves a complex and poorly understood molecular and biological interplay between the mother and fetus (Crider *et al.*, 2005), there is increasing evidence that infection and the inflammatory response contribute to the etiology of PTB (Goldenberg *et al.*, 2000). Genetic susceptibility factors in immune response genes have been investigated, particularly cytokine polymorphisms which are known to be proinflammatory. Cytokines, such as interleukin (IL)-1 (Romero *et al.*, 1989a; Hillier *et al.*, 1993)

and IL-6 (Greig *et al.*, 1997), are responsible for the onset of premature labor and parturition. The IL-1 family consists of three subtypes (gene symbols are in parentheses): IL-1α (*IL1A*), IL-1β (*IL1B*) and the specific receptor antagonist, IL-1Ra (*IL1RN*). The two forms of IL-1, α and β, are products of distinct genes and thus have different amino acid sequences; however, they have similar three-dimensional structures, interact with similar receptors and share biological activities (Dinarello, 1991). IL-1α and IL-1β stimulate prostaglandin E2 synthesis by the amnion (Romero *et al.*, 1989b). Single-nucleotide polymorphisms (SNPs) include −889C/T in the transcriptional regulatory region and +4845G/T (A114S) in the coding region of *IL1A*, and −511C/T and −31C/T in the transcriptional regulatory region of *IL1B*. Evidence suggests that to a certain extent these polymorphisms cause functional changes. The *IL1A* −889TT genotype significantly increases the transcriptional activity of *IL1A* compared with the −889CC genotype (Dominici *et al.*, 2002). Peripheral blood mononuclear cells with the *IL1B* −511T genotype tend to produce slightly higher IL-1β levels than peripheral blood mononuclear cells with the *IL1B* −511C genotype (Santtila *et al.*, 1998). And recently, it was reported that SNPs in the *IL1B* promoter region, containing −511C/T and −31C/T polymorphisms, affect transcription according to haplotype context (Chen *et al.*, 2006).

The *IL1A* and *IL1B* polymorphisms are associated with inflammatory diseases, such as periodontal and cardiovascular diseases (Kornman *et al.*, 1997; Kornman and Duff, 2001). These polymorphisms may also influence the onset of premature labor. One study has reported that the fetal *IL1B*+3953 polymorphism is associated with PTB in the African American population (Genc *et al.*, 2002). However, no studies have detected significant associations between maternal *IL1A* or *IL1B* polymorphisms and PTB (Annells *et al.*, 2004; Moore *et al.*, 2004; Engel *et al.*, 2005; Edwards *et al.*, 2006).

We examined the relationships of common polymorphisms in proinflammatory cytokines with PTB and LBW in a cohort of women enrolled in the present study. In this report, we describe the relationship between six polymorphisms in proinflammatory cytokine genes (i.e. *IL1A*, *IL1B*, *IL2* and *IL6*) and PTB and LBW. Thus, the aim of the present study was to elucidate the association of proinflammatory cytokine polymorphisms with PTB, LBW and intrauterine growth restriction (IUGR) in a Japanese population.

Materials and Methods

Subjects

This birth cohort study was performed in the city of Sapporo, Japan, as described in detail previously (Sata *et al.*, 2006). We recruited the women during scheduled appointments at the Hokkaido University Hospital, a tertiary hospital in the region, for a medical examination one month after delivery between November 2001 and April 2005. A consecutive series of 466 women who had singleton deliveries were studied. There were 52 women who had autoimmune disease, anti-phospholipid syndrome, congenital thrombophilia, gestational diabetes mellitus or who delivered malformed infants in the index pregnancies, and were therefore excluded from the study. Altogether, 414 eligible women, 18–44 years of age, were analyzed in the present study. All the women were residents of Sapporo or the surrounding areas in Japan and all were native Japanese. In recent years, this geographical region has had little immigration by different ethnic groups. The subjects included 73 mothers who had a PTB and 341 mothers who had a term birth (TB, a birth between 37 and 41 weeks of gestation). PTBs were also subdivided according to gestational age: 12.3% of PTBs occurred at <28 weeks (extreme prematurity), 15.1% at 28–31 weeks (severe prematurity), 27.4% at 32–33 weeks (moderate prematurity) and 45.2% at 34–36 weeks (near term). The overall rates of PTB and LBW were approximately 18 and 25% in this population, as compared with the national average of 5.7 and 9.4%, respectively (Mothers' and Children's Health and Welfare Association, 2007). Such high rates of PTB and LBW in tertiary hospitals have been observed elsewhere in Japan (Kawamata *et al.*, 2007). The study was conducted with informed consent of all subjects and was approved by the Institutional Ethical Board for Human Gene and Genome Studies of Hokkaido University Graduate School of Medicine.

The characteristics of the women, divided according to their gestational age at birth are shown in Table I. The maternal mean (SD) age at the time of delivery and gestational age were 31.4 (5.2) years and 37.9 (2.9) weeks, respectively. There were 50 women (12.9%) who continued smoking cigarettes and 80 women (21.1%) who continued alcohol consumption at least once a month throughout pregnancy. The mean (SD) birthweight of the newborns was 2.78 (0.63) kg. We found no significant differences in maternal age at birth, parity, cigarette smoking status, alcohol use during pregnancy or infant gender between the mothers who had a PTB and the mothers who had a TB. The etiologies of the 73 cases of PTB were classified as spontaneous ($n = 43$) or medically indicated ($n = 30$), according to the published guidelines (Pennell *et al.*, 2007). Spontaneous PTB were comprised of preterm labor leading to PTB (idiopathic PTB, 51.2%) and preterm premature rupture of the membranes (PPROM, 48.8%).

Selection and determination of proinflammatory cytokine polymorphisms

We selected six common polymorphisms of proinflammatory cytokines, with minor allele frequencies of at least 10% in a Japanese population according to a SNP database, such as the International Hapmap Project (Hapmap Homepage, 2006). Each polymorphism predicted a functional change because it was located in the coding region with an amino acid substitution or in the 5′-flanking region, especially in the transcription factor binding site (Table II).

Genomic DNA was extracted from lymphocytes of peripheral blood using the QIAamp or EZ1 DNA blood kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. We genotyped each polymorphism by allelic discrimination using fluorogenic probes and the 5′ nuclease (TaqMan) assay, as previously described (Sata *et al.*, 2006). TaqMan® SNP Genotyping Assays for each polymorphism were obtained from Applied Biosystems (Foster City, CA, USA). All probe-primer sets were designed to function using universal reaction and cycling conditions. Genotyping was performed in 10 μl reactions containing approximately 40 ng genomic DNA, 0.5 μl 20× TaqMan® SNP Genotyping Assay Mix (consisting of unlabeled PCR primers, and FAM and VIC dye-labeled TaqMan® MGB probes) and 5.0 μl of 2× Taqman® Universal PCR Master Mixture. Real-time PCR was performed on a 7500 Real-time PCR System (Applied Biosystems) using a protocol consisting of incubation at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation at 92°C for 15 s and annealing/extension at 60°C for 1 min. FAM and VIC fluorescence levels of PCR products were measured at 60°C for 1 min, resulting in the clear identification of all three genotypes in these polymorphisms on a two-dimensional graph. We confirmed no contamination by using a no template control. The samples that had not been clearly classified into genotypes the first time and a small number of other samples were analyzed using positive controls as well as a no template control. Finally, at least two researchers or

Table I Characteristics of Japanese mothers, by gestational age

	All subjects ($n = 414$)	Gestational age, <37 weeks ($n = 73$)	Gestational age, ≥37 weeks ($n = 341$)	*P*-value[a]
Age (%)				
≤29 years	35.3	27.4	37.0	0.25
30–39 years	58.5	67.1	56.6	
≥40 years	6.3	5.5	6.5	
Parity (%)				
0	53.9	58.9	52.8	0.34
≥1	46.1	41.1	47.2	
Smoking status during pregnancy (%)[b]				
Current smoker	12.9	13.2	12.9	0.93
Non-smoker	87.1	86.8	87.1	
Alcohol use during pregnancy (%)[c]				
Current drinker	21.1	14.9	22.4	0.18
Non-drinker	78.9	85.1	77.6	
Infant gender (%)				
Male	54.3	45.2	56.3	0.08
Female	45.7	54.8	46.7	
Intrauterine growth retardation (%)				
<10th percentile	14.7	20.5	13.5	0.12
≥10th percentile	85.3	79.5	86.5	

[a]Chi-square test; [b]Here 27 mothers (5 mothers with PTB and 22 mothers with TB) had missing data; [c]Here 34 mothers (6 mothers with PTB and 28 mothers with TB) had missing data. PTB, preterm birth; TB, term birth.

Table II Proinflammatory cytokine polymorphisms and allele frequencies in Japanese mothers in Sapporo City

Gene	dbSNP ID	SNP region		Allele frequency
IL1A	rs1800587	−889C>T	5′ UTR	C = 0.91, T = 0.09
	rs17561	+4845G>T	A114S	G = 0.90, T = 0.10
IL1B	rs16944	−511C>T	5′ UTR	C = 0.50, T = 0.50
	rs1143627	−31C>T	5′ UTR, tfbs	C = 0.50, T = 0.50
IL2	rs2069762	−384 T>G	5′ UTR, tfbs	T = 0.67, G = 0.33
IL6	rs1800796	−634C>G	5′ UTR	C = 0.80, G = 0.20

5′ UTR, 5′ untranslational or flanking region; tfbs, transcription factor binding site.

technicians confirmed that all the samples were clearly classified into genotypes.

Statistical analyses

The differences in frequency of each characteristic between the PTB and TB groups were examined by a chi-square test. An unconditional logistic regression model was used to evaluate the associations between maternal proinflammatory cytokine genotypes and PTB, LBW or IUGR (<10th percentile of birthweights or less than the mean 1.5 SD of the birthweight). We calculated non-adjusted and adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for each genotype together with dominant and recessive genotype models by unconditional logistic regression analysis. As confounding factors, maternal age at birth (continuous), parity (never = 0 and any = 1), cigarette smoking status (none = 0 and continuous = 1), alcohol use (none = 0 and continuous = 1) during pregnancy and infant gender (female=0 and male=1) were considered (Table I). Bonferroni correction was performed as a multiple comparison test. Hardy–Weinberg equilibrium analyses were performed to compare observed and expected genotype frequencies using a chi-square test. The haplotype was analyzed using Haploview, version 4.0, based on the expectation-maximization algorithm (Barrett *et al.*, 2005), and linkage disequilibrium between loci was measured using Lewontin's D′ (Hedrick, 1987). Statistical analyses were performed using SPSS software for Windows, version 15.0 (SPSS, Chicago, IL, USA).

Results

The frequencies of the common inflammatory cytokine genotypes were compared between 73 mothers who had a PTB and 341 control

Table III Associations of maternal proinflammatory cytokine polymorphisms with PTB in Japanese mothers in Sapporo City

	Gestational age, ≥37 weeks (n = 341)	Gestational age, <37 weeks (n = 73)	Adjusted OR† (95% CI)	Spontaneous PTB (n = 43)	Adjusted OR† (95% CI)	Idiopathic PTB (n = 22)	Adjusted OR† (95% CI)	PPROM (n = 21)	Adjusted OR† (95% CI)
IL1A −889C/T									
CC	290 (85.0)	50 (67.1)	1.0 (Ref.)	30 (69.8)	1.0 (Ref.)	11 (50.0)	1.0 (Ref.)	19 (90.5)	1.0 (Ref.)
CT	49 (14.4)	22 (31.5)	**2.5** (1.4–4.8)**	12 (27.9)	2.1 (1.0–4.7)	10 (45.5)	**4.9** (1.8–13.5)**	2 (9.5)	0.6 (0.1–2.8)
TT	2 (0.6)	1 (1.4)	1.8 (0.2–20.6)	1 (2.3)	3.4 (0.3–41.7)	1 (4.5)	11.1 (0.8–154.1)	0 (0.0)	0 (−)
Dominant model									
CC	290 (85.0)	50 (67.1)	1.0 (Ref.)	30 (69.8)	1.0 (Ref.)	11 (50.0)	1.0 (Ref.)	19 (90.5)	1.0 (Ref.)
CT+TT	51 (15.0)	23 (2.9)	**2.5** (1.3–4.6)**	13 (30.2)	**2.2* (1.0–4.8)**	11 (50.0)	**5.2** (2.0–13.9)**	2 (9.5)	0.6 (0.1–2.7)
Recessive model									
CC+CT	339 (99.4)	72 (98.6)	1.0 (Ref.)	42 (97.7)	1.0 (Ref.)	21 (95.5)	1.0 (Ref.)	21 (100.0)	1.0 (Ref.)
TT	2 (0.6)	1 (1.4)	1.4 (0.1–16.0)	1 (23)	2.9 (0.2–34.1)	1 (4.5)	6.3 (0.5–8.25)	0 (0.0)	0.0 (−)
IL1A +4845G/T (A114S)									
GG	288 (84.5)	48 (65.8)	1.0 (Ref.)	30 (69.8)	1.0 (Ref.)	11 (50.0)	1.0 (Ref.)	19 (90.5)	1.0 (Ref.)
GT	50 (14.7)	25 (32.9)	**2.4** (1.3–4.4)**	12 (27.9)	2.0 (0.9–4.4)	10 (45.5)	**4.5** (1.7–12.2)**	2 (9.5)	0.6 (0.1–2.7)
TT	3 (0.9)	1 (1.4)	1.4 (0.1–14.7)	1 (2.3)	2.7 (0.3–28.5)	1 (4.5)	8.9 (0.7–106.9)	0 (0.0)	0.0 (−)
Dominant model									
GG	288 (84.5)	48 (65.8)	1.0 (Ref.)	30 (69.8)	1.0 (Ref.)	11 (50.0)	1.0 (Ref.)	19 (90.5)	1.0 (Ref.)
GT+TT	53 (15.6)	25 (34.3)	**2.3**(1.2–4.2)**	13 (30.2)	2.0 (0.9–4.4)	11 (50.0)	**4.7**(1.8–12.5)**	2 (9.5)	0.6 (0.1–2.5)
Recessive model									
GG+GT	338 (99.1)	72 (98.6)	1.0 (Ref.)	42 (97.7)	1.0 (Ref.)	21 (95.5)	1.0 (Ref.)	21 (100.0)	1.0 (Ref.)
TT	3 (0.9)	1 (1.4)	1.1 (0.1–11.5)	1 (2.3)	2.3 (0.2–23.8)	1 (4.5)	5.2 (0.5–60.2)	0 (0.0)	0.0 (−)
IL1B −511C/T									
CC	86 (25.2)	26 (35.6)	1.0 (Ref.)	15 (34.9)	1.0 (Ref.)	9 (40.9)	1.0 (Ref.)	6 (28.6)	1.0 (Ref.)
CT	162 (47.5)	27 (37.0)	0.5 (0.3–1.0)	15 (34.9)	0.5 (0.3–1.2)	6 (27.3)	0.4 (0.1–1.2)	9 (42.9)	0.8 (0.3–2.3)
TT	93 (27.3)	20 (27.4)	0.7 (0.3–1.3)	13 (30.2)	0.7 (0.3–1.6)	7 (31.8)	0.5 (0.1–1.6)	6 (28.6)	1.0 (0.3–3.3)
Dominant model									
CC	86 (25.2)	26 (35.6)	1.0 (Ref.)	15 (34.9)	1.0 (Ref.)	9 (40.9)	1.0 (Ref.)	6 (28.6)	1.0 (Ref.)
CT+TT	255 (74.8)	47 (64.4)	0.6 (0.3–1.0)	28 (65.1)	0.6 (0.3–1.2)	13 (59.1)	0.4 (0.2–1.1)	15 (71.5)	0.9 (0.3–2.3)
Recessive model									
CC+CT	248 (72.7)	53 (72.6)	1.0 (Ref.)	30 (69.8)	1.0 (Ref.)	15 (68.2)	1.0 (Ref.)	15 (71.5)	1.0 (Ref.)
TT	93 (27.3)	20 (27.4)	0.9 (0.5–1.7)	13 (30.2)	1.0 (0.4–2.1)	7 (31.8)	0.7 (0.2–2.3)	6 (28.6)	1.2 (0.4–3.2)
IL1B −31C/T									
CC	91 (26.7)	17 (23.3)	1.0 (Ref.)	11 (25.6)	1.0 (Ref.)	7 (31.8)	1.0 (Ref.)	4 (19.0)	1.0 (Ref.)
CT	166 (48.7)	30 (41.1)	1.1 (0.5–2.2)	17 (39.5)	1.1 (0.5–2.7)	6 (27.3)	0.9 (0.2–3.2)	11 (52.4)	1.4 (0.4–4.7)
TT	84 (24.6)	26 (35.6)	1.9 (0.9–3.8)	15 (34.9)	1.9 (0.7–4.6)	9 (40.9)	2.2 (0.7–7.5)	6 (28.6)	1.5 (0.4–5.5)

Dominant model									
CC	91 (26.7)	17 (23.3)	1.0 (Ref.)	11 (25.6)	1.0 (Ref.)	7 (31.8)	1.0 (Ref.)	4 (19.0)	1.0 (Ref.)
CT+TT	250 (73.3)	56 (76.7)	1.4 (0.7–2.6)	32 (74.4)	1.4 (0.6–3.2)	15 (68.2)	1.4 (0.4–4.2)	17 (81.0)	1.4 (0.5–4.5)
Recessive model									
CC+CT	257 (75.4)	47 (64.4)	1.0 (Ref.)	28 (65.1)	1.0 (Ref.)	13 (59.1)	1.0 (Ref.)	15 (71.5)	1.0 (Ref.)
TT	84 (24.6)	26 (35.6)	1.7 (1.0–3.1)	15 (34.9)	1.7 (0.9–3.4)	9 (40.9)	2.4 (0.9–6.2)	6 (28.6)	1.2 (0.4–3.2)
IL2 −384 T/G									
TT	144 (42.2)	32 (43.8)	1.0 (Ref.)	20 (46.5)	1.0 (Ref.)	10 (45.5)	1.0 (Ref.)	10 (47.6)	1.0 (Ref.)
TG	166 (48.7)	36 (49.4)	0.8 (0.5–1.5)	21 (48.8)	0.8 (0.4–1.6)	12 (54.5)	0.9 (0.3–2.3)	9 (42.9)	0.7 (0.3–1.8)
GG	31 (9.1)	5 (6.8)	0.8 (0.3–2.2)	2 (4.7)	0.5 (0.1–2.1)	0 (0.0)	0.0 (−)	2 (9.5)	0.8 (0.2–4.1)
Dominant model									
TT	144 (42.2)	32 (43.8)	1.0 (Ref.)	20 (46.5)	1.0 (Ref.)	10 (45.5)	1.0 (Ref.)	10 (47.6)	1.0 (Ref.)
TG+GG	197 (57.8)	41 (56.2)	0.8 (0.5–1.4)	23 (53.5)	0.7 (0.4–1.4)	12 (54.5)	0.7 (0.3–1.9)	11 (52.4)	0.7 (0.3–1.7)
Recessive model									
TT+TG	310 (90.9)	68 (93.2)	1.0 (Ref.)	41 (95.3)	1.0 (Ref.)	22 (100.0)	1.0 (Ref.)	19 (90.5)	1.0 (Ref.)
GG	31 (9.1)	5 (6.8)	0.8 (0.3–2.3)	2 (4.7)	0.5 (0.1–2.3)	0 (0.0)	0.0 (−)	2 (9.5)	1.0 (0.2–4.6)
IL6 −634C/G									
CC	222 (65.1)	47 (64.4)	1.0 (Ref.)	28 (65.1)	1.0 (Ref.)	15 (68.2)	1.0 (Ref.)	13 (61.9)	1.0 (Ref.)
CG	105 (30.8)	22 (30.1)	1.0 (0.5–1.8)	13 (30.2)	1.0 (0.5–2.1)	5 (22.7)	0.7 (0.2–2.1)	8 (38.1)	1.4 (0.5–3.5)
GG	14 (4.1)	4 (5.5)	1.7 (0.5–5.5)	2 (4.7)	1.5 (0.3–7.4)	2 (9.1)	3.1 (0.6–16.4)	0 (0.0)	0.0 (−)
Dominant model									
CC	222 (65.1)	47 (64.4)	1.0 (Ref.)	28 (65.1)	1.0 (Ref.)	15 (68.2)	1.0 (Ref.)	13 (61.9)	1.0 (Ref.)
CG+GG	119 (34.9)	26 (35.6)	1.1 (0.6–1.9)	15 (34.9)	1.1 (0.5–2.2)	7 (31.8)	0.9 (0.3–2.4)	8 (38.1)	1.2 (0.5–3.2)
Recessive model									
CC+CG	327 (95.9)	69 (94.5)	1.0 (Ref.)	41 (95.3)	1.0 (Ref.)	20 (90.9)	1.0 (Ref.)	21 (100.0)	1.0 (Ref.)
GG	14 (4.1)	4 (5.5)	1.7 (0.5–5.5)	2 (4.7)	1.5 (0.3–7.3)	2 (9.1)	3.5 (0.7–18.0)	0 (0.0)	0.0 (−)

[1]Unconditional logistic regression analysis adjusted for maternal age at birth (continuous), parity (never=0, any=1), smoking status (none=0, continuous=1) and alcohol use (none=0, continuous=1) during pregnancy and infant gender (female=0, male=1). Bold values indicate statistical significance. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. CI, confidence interval; OR, odds ratio; PPROM, preterm premature rupture of the membranes; PTB, preterm birth.

mothers who had a TB in a Japanese population (Table III). The distribution of genotypes in each group was in Hardy–Weinberg equilibrium (chi-square test, $P > 0.05$). We found significantly increased risks for PTB in the *IL1A* −889C/T and +4845G/T groups. In this analysis, we considered maternal age at birth, parity, cigarette smoking status, alcohol use during pregnancy and infant gender as confounding factors (Table III). However, the adjusted results were nearly the same as the crude results. The risk of a PTB significantly increased in women carrying the *IL1A* −889T allele (CC genotype [reference]; CT genotype, OR: 2.5; 95% CI: 1.4–4.8, $P < 0.01$; CT+TT genotypes [dominant genotype model], OR: 2.5, 95% CI: 1.3–4.6, $P < 0.01$). Similarly, the risk of a PTB significantly increased in women carrying the *IL1A* +4845T allele (GG genotype [reference]; GT genotype, OR: 2.4, 95% CI: 1.3–4.4, $P < 0.01$; GT+TT genotypes [dominant genotype model], OR: 2.3, 95% CI: 1.2–4.2, $P < 0.01$). However, we found no significant difference between the distributions of other genotypes in the two groups ($P > 0.05$).

We evaluated the risk of PTB in the subgroups of patients with spontaneous PTB classified into idiopathic PTB and PPROM; (Table III). We found significant increased risks for both idiopathic PTB and medically indicated PTB for those with *IL1A* −889C/T and +4845G/T polymorphisms, whereas no significant increased risks existed for PPROM for those with these polymorphisms.

There were two haplotype blocks identified in the present study: (i) *IL1A* −889C/T and +4845G/T, located on chromosome 2q14 (Block 1), and (ii) *IL1B* −511C/T and −31C/T, located on chromosome 2q14 (Block 2, Table IV). The highest degree of linkage disequilibrium was observed between polymorphisms *IL1A* −889C/T and +4845G/T ($D' = 0.98$) and between polymorphisms *IL1B* −511C/T and −31C/T ($D' = 0.98$). The frequency of the *IL1A* TT haplotype in mothers who had a PTB was significantly higher than those who had a TB ($P = 0.0003$), whereas the frequency of the *IL1A* CG haplotype in mothers who had a PTB was significantly lower compared with those who had a TB ($P = 0.0006$). The permutation test was conducted 10 000 times and the statistical significance persisted ($P = 0.01$ for both). The risk of a PTB significantly increased in women carrying the *IL1A* TT haplotype (non-carriers of TT haplotype [reference]; carriers of TT haplotype, OR: 2.5; 95% CI: 1.5–4.2, $P < 0.001$), whereas the risk of a PTB significantly decreased in women carrying the *IL1A* CG haplotype (non-carriers of CG haplotype [reference]; carriers of CG haplotype, OR: 0.4; 95% CI: 0.3–0.7, $P < 0.001$). On the other hand, there were no significant changes in any haplotype frequencies of the *IL1B* block.

The frequencies of the inflammatory cytokine genotypes were also compared between 104 mothers who had a LBW infant and 310 control mothers in a Japanese population (Table V). We found significantly increased risks for LBW infants in *IL1A* −889C/T and +4845G/T polymorphisms. However, there were no significant differences in the distribution of other genotypes within the two groups ($P > 0.05$). We also evaluated the risk of IUGR using two different definitions of IUGR, which are used in Japan, as shown in Table V. We did not find any significantly increased nor decreased risks for IUGR with any polymorphism.

Discussion

We assessed the possible role of common proinflammatory cytokine genes observed in a Japanese population in the present study. To the best of our knowledge, this is the first report demonstrating significant associations between maternal *IL1A* polymorphisms and PTB. The risk of PTB was significantly increased in women carrying the *IL1A* −889T or the *IL1A* +4845T alleles. We confirmed these significant risks in cases with idiopathic PTB, where the causes are closely associated with premature labor due to the production of inflammatory cytokines (Keelan *et al.*, 2003). We found that the *IL1A* locus was associated with idiopathic PTB, but not with PPROM. It is suggested that a specific mechanism, such as an abnormality of IL1-α production, might lead to idiopathic PTB, which is quite different from the etiology of PPROM. Such etiological differences between idiopathic PTB and PPROM have been reported: the risk factors for idiopathic PTB include personal obstetrical history, social factors and lifestyle, whereas those for PPROM include disadvantaged population, African American ethnicity and infection (Moutquin, 2003). Thus, births that follow spontaneous preterm labour and PPROM are regarded as a syndrome resulting from multiple causes, including

Table IV Haplotype association with PTB in Japanese mothers in Sapporo City

	Overall ($n = 828$)	Gestational age, ≥37 weeks ($n = 682$)	Gestational age, <37 weeks ($n = 146$)	Chi-square	*P*-value	Permutation test, *P*-value†	OR‡ (95% CI)
IL1A −889C/T, +4845G/T (A1145)							
CG	90	91.6	82.2	11.9	**0.0006*** **	**0.01***	**0.4*** (0.3–0.7)**
TT	9.3	7.6	17.1	12.7	**0.0003*** **	**0.01***	**2.5*** (1.5–4.2)**
CT	0.6	0.6	0.7	0.02	0.89	1	1.2 (0.1–10.5)
TG	0.1	0.1	0	0.21	0.65	1	–
IL1B −511C/T, −31C/T							
CT	49.4	48.4	54.1	1.58	0.21	0.74	1.3 (0.9–1.8)
TC	49.3	50.4	43.8	2.1	0.15	0.61	0.8 (0.5–1.1)
TT	0.8	0.6	2.1	3.08	0.08	0.45	3.6 (0.8–16.1)
CC	0.5	0.6	0	0.86	0.35	0.93	–

†*P*-values based on 10 000 permutations and represent the comparison of individual haplotype frequencies between cases and controls; ‡OR and 95% CI calculated comparing carriers of a given haplotype to noncarriers of that haplotype. Bold values indicate statistical significance. *$P < 0.05$; ***$P < 0.001$. CI, confidence interval; OR, odds ratio.

Table V Associations of maternal proinflammatory cytokine polymorphisms with low birthweight and intrauterine growth restriction in Japanese mothers in Sapporo City

	Birthweight ≥2500 g (n = 310)	Birthweight <2500 g (n = 104)	Adjusted OR[†] (95% CI)	Birthweight ≥10th percentile (N = 353)	Birthweight <10th percentile (n = 61)	Adjusted OR[†] (95% CI)	Birthweight ≥ mean − 1.5 SD (n = 375)	Birthweight < mean − 1.5 SD (n = 39)	Adjusted OR[†] (95% CI)
IL1A −889C/T									
CC	264 (85.2)	75 (72.1)	1.0 (Ref.)	291 (82.4)	48 (78.7)	1.0 (Ref.)	309 (82.4)	30 (76.9)	1.0 (Ref.)
CT	44 (14.2)	28 (26.9)	**1.9* (1.0–3.4)**	59 (16.7)	13 (21.3)	1.2 (0.6–2.6)	63 (16.8)	9 (23.1)	1.5 (0.6–3.6)
TT	2 (0.6)	1 (1.0)	1.1 (0.1–12.2)	3 (0.8)	0 (0.0)	0 (−)	3 (0.8)	0 (0.0)	0 (−)
Dominant model									
CC	264 (85.2)	75 (72.1)	1.0 (Ref.)	291 (82.4)	48 (78.7)	1.0 (Ref.)	309 (82.4)	30 (76.9)	1.0 (Ref.)
CT+TT	46 (14.8)	29 (27.9)	**1.8* (1.0–3.2)**	62 (17.6)	13 (21.3)	1.2 (0.5–2.5)	66 (17.6)	9 (23.1)	1.5 (0.6–3.5)
Recessive model									
CC+CT	308 (99.4)	103 (99.0)	1.0 (Ref.)	350 (99.2)	61 (100.0)	1.0 (Ref.)	372 (99.2)	39 (100.0)	1.0 (Ref.)
TT	2 (0.6)	1 (1.0)	0.9 (0.1–10.4)	3 (0.8)	0 (0.0)	0 (−)	3 (0.8)	0 (0.0)	0 (−)
IL1A +4845G/T (A114S)									
GG	263 (84.8)	73 (70.2)	1.0 (Ref.)	289 (81.9)	47 (77.0)	1.0 (Ref.)	306 (81.6)	30 (76.9)	1.0 (Ref.)
GT	44 (14.2)	30 (28.8)	**2.0* (1.1–3.6)**	61 (17.3)	13 (21.3)	1.2 (0.5–2.5)	66 (17.6)	8 (20.5)	1.2 (0.5–3.0)
TT	3 (1.0)	1 (1.0)	0.9 (0.1–9.1)	3 (0.8)	1 (1.6)	2.8 (0.3–29.2)	3 (0.8)	1 (2.6)	5.3 (0.5–57.8)
Dominant model									
GG	263 (84.8)	73 (70.2)	1.0 (Ref.)	289 (81.9)	47 (77.0)	1.0 (Ref.)	306 (81.6)	30 (76.9)	1.0 (Ref.)
GT+TT	47 (15.2)	31 (29.8)	**1.9* (1.1–3.4)**	64 (18.1)	14 (22.9)	1.2 (0.6–2.6)	69 (18.4)	9 (23.1)	1.4 (0.6–3.2)
Recessive model									
GG+GT	307 (99.0)	103 (99.0)	1.0 (Ref.)	350 (99.2)	60 (98.4)	1.0 (Ref.)	372 (99.2)	39 (100.0)	1.0 (Ref.)
TT	3 (1.0)	1 (1.0)	0.7 (0.1–7.6)	3 (0.8)	1 (1.6)	2.7 (0.3–27.9)	3 (0.8)	0 (0.0)	5.1 (0.5–54.4)
IL1B −511C/T									
CC	78 (25.2)	34 (32.7)	1.0 (Ref.)	90 (25.5)	22 (36.1)	1.0 (Ref.)	100 (26.7)	12 (30.8)	1.0 (Ref.)
CT	151 (48.7)	38 (36.5)	0.7 (0.4–1.2)	168 (47.6)	21 (34.4)	0.7 (0.4–1.3)	173 (46.1)	16 (41.0)	1.0 (0.5–2.2)
TT	81 (26.1)	32 (30.8)	0.9 (0.5–1.6)	95 (26.9)	18 (29.5)	0.7 (0.3–1.4)	102 (27.2)	11 (28.2)	0.8 (0.4–2.0)
Dominant model									
CC	78 (25.2)	34 (32.7)	1.0 (Ref.)	90 (25.5)	22 (36.1)	1.0 (Ref.)	100 (26.7)	12 (30.8)	1.0 (Ref.)
CT+TT	232 (74.8)	70 (67.3)	0.8 (0.5–1.3)	263 (74.5)	39 (63.9)	0.7 (0.4–1.3)	275 (73.3)	27 (69.2)	1.0 (0.5–1.9)
Recessive model									
CC+CT	229 (73.9)	72 (69.2)	1.0 (Ref.)	258 (73.1)	43 (70.5)	1.0 (Ref.)	273 (72.8)	28 (71.8)	1.0 (Ref.)
TT	81 (26.1)	32 (30.8)	1.1 (0.6–1.7)	95 (26.9)	18 (29.5)	0.9 (0.5–1.6)	102 (27.2)	11 (28.2)	0.8 (0.4–1.7)

Continued

Table V *Continued*

	Birthweight ≥2500 g (n = 310)	Birthweight <2500 g (n = 104)	Adjusted OR† (95% CI)	Birthweight ≥10th percentile (N = 353)	Birthweight <10th percentile (n = 61)	Adjusted OR† (95% CI)	Birthweight ≥ mean − 1.5 SD (n = 375)	Birthweight < mean − 1.5 SD (n = 39)	Adjusted OR† (95% CI)
IL1B −31C/T									
CC	79 (25.5)	29 (27.9)	1.0 (Ref.)	90 (25.5)	18 (29.5)	1.0 (Ref.)	97 (25.9)	11 (28.2)	1.0 (Ref.)
CT	155 (50.0)	41 (39.4)	0.9 (0.5–1.7)	175 (49.6)	21 (34.4)	0.7 (0.3–1.5)	180 (48.0)	16 (41.0)	0.9 (0.4–2.2)
TT	76 (24.5)	34 (32.7)	1.4 (0.7–2.6)	88 (24.9)	22 (36.1)	1.3 (0.6–2.8)	98 (26.1)	12 (30.8)	1.2 (0.4–3.0)
Dominant model									
CC	79 (25.5)	29 (27.9)	1.0 (Ref.)	90 (25.5)	18 (29.5)	1.0 (Ref.)	97 (25.9)	11 (28.2)	1.0 (Ref.)
CT+TT	231 (74.5)	75 (72.1)	1.1 (0.6–1.9)	263 (74.5)	43 (70.5)	0.9 (0.5–1.8)	278 (74.1)	28 (71.8)	1.0 (0.4–2.2)
Recessive model									
CC+CT	234 (75.5)	70 (67.3)	1.0 (Ref.)	265 (75.1)	39 (63.9)	1.0 (Ref.)	277 (73.9)	27 (69.2)	1.0 (Ref.)
TT	76 (24.5)	34 (32.7)	1.4 (0.9–2.4)	88 (24.9)	22 (36.1)	1.7 (0.9–3.1)	98 (26.1)	12 (30.8)	1.2 (0.6–2.6)
IL2 −384 T/G									
TT	135 (43.5)	41 (39.4)	1.0 (Ref.)	148 (41.9)	28 (45.9)	1.0 (Ref.)	159 (42.4)	17 (43.6)	1.0 (Ref.)
TG	145 (46.8)	57 (54.8)	1.2 (0.7–2.0)	173 (49.0)	29 (47.5)	0.9 (0.5–1.6)	183 (48.8)	19 (48.7)	0.9 (0.4–1.8)
GG	30 (9.7)	6 (5.8)	0.7 (0.3–1.9)	32 (9.1)	4 (6.6)	0.7 (0.2–2.3)	33 (8.8)	3 (7.7)	0.9 (0.2–3.2)
Dominant model									
TT	135 (43.5)	41 (39.4)	1.0 (Ref.)	148 (41.9)	28 (45.9)	1.0 (Ref.)	159 (42.4)	17 (43.6)	1.0 (Ref.)
TG+GG	175 (56.5)	63 (60.6)	1.1 (0.7–1.8)	205 (58.1)	33 (54.1)	0.9 (0.5–1.5)	216 (57.6)	23 (56.4)	0.9 (0.4–1.8)
Recessive model									
TT+TG	280 (90.3)	98 (94.2)	1.0 (Ref.)	321 (90.9)	57 (93.4)	1.0 (Ref.)	342 (91.2)	36 (92.3)	1.0 (Ref.)
GG	30 (9.7)	6 (5.8)	0.7 (0.3–1.7)	32 (9.1)	4 (6.6)	0.8 (0.3–2.4)	33 (8.8)	3 (7.7)	0.9 (0.3–3.3)
IL6 −634C/G									
CC	203 (65.5)	66 (63.5)	1.0 (Ref.)	230 (65.2)	39 (63.9)	1.0 (Ref.)	243 (64.8)	26 (66.7)	1.0 (Ref.)
CG	92 (29.7)	35 (33.7)	1.1 (0.7–1.9)	106 (30.0)	21 (34.4)	1.1 (0.6–2.0)	114 (30.4)	13 (33.3)	1.1 (0.5–2.3)
GG	15 (4.8)	3 (2.9)	0.7 (0.2–2.6)	17 (4.8)	1 (1.6)	0.3 (0.04–2.6)	18 (4.8)	0 (0.0)	0 (−)
Dominant model									
CC	203 (65.5)	66 (63.5)	1.0 (Ref.)	230 (65.2)	39 (63.9)	1.0 (Ref.)	243 (64.8)	26 (66.7)	1.0 (Ref.)
CG+GG	107 (34.5)	38 (36.5)	1.1 (0.6–1.7)	123 (34.8)	22 (36.1)	1.0 (0.5–1.8)	132 (35.2)	13 (33.3)	0.9 (0.4–1.9)
Recessive model									
CC+CG	295 (95.2)	101 (97.1)	1.0 (Ref.)	336 (95.2)	60 (98.4)	1.0 (Ref.)	357 (95.2)	39 (100.0)	1.0 (Ref.)
GG	15 (4.8)	3 (2.9)	0.7 (0.2–2.5)	17 (4.8)	1 (1.6)	0.3 (0.4–2.5)	18 (4.8)	0 (0.0)	0 (−)

†Unconditional logistic regression analysis adjusted for maternal age at birth (continuous), parity (never=0, any=1), smoking status (none=0, continuous=1) and alcohol use (none=0, continuous=1) during pregnancy and infant gender (female=0, male=1). Bold face indicates statistical significance. *$P < 0.05$; **$P < 0.01$. CI, confidence interval; OR, odds ratio.

infection or inflammation, vascular disease and uterine overdistension (Goldenberg *et al.*, 2008). In the present study, however, there was no significant difference in the distribution of gestational age between idiopathic PTB and PPROM.

The *IL1A* −889C/T polymorphism is associated with susceptibility to several diseases, such as juvenile rheumatoid arthritis (McDowell *et al.*, 1995), osteoarthritis (Loughlin *et al.*, 2002), periodontal disease (Kornman *et al.*, 1997) and Alzheimer's disease (Rogers, 2000). These studies have suggested that the *IL1A* −889T allele alters the transcriptional ability of *IL1A*, resulting in an aberrant production of IL-1α in these diseases. The *IL1A* −889TT genotype creates a consensus site for the transcription factor, Skn-1, and is associated with a significant increase in promoter activity compared with the −889CC genotype (Hulkkonen *et al.*, 2000). A slight increase in IL-1α mRNA and protein levels in the plasma has been detected in a carrier of the *IL1A* −889TT genotype (Dominici *et al.*, 2002). Regarding the other polymorphism, one of the risk factors of PTB and periodontal disease is also related to the polymorphisms of *IL1A* +4845G/T (Offenbacher *et al.*, 2001; Thomson *et al.*, 2001; Li *et al.*, 2004). *IL1A* +4845T (114S) is associated with risks of inflammatory disorders, reflecting increased IL-1α production in carriers of this allele and the *IL1A* −889T allele (Thomson *et al.*, 2001).

In the present study, the frequency of the *IL1A* TT haplotype in mothers who had a PTB was remarkably higher than in those who had a TB ($P < 0.001$), whereas the CG haplotype in mothers who had a PTB was remarkably lower than in those who had a TB ($P < 0.001$; Table IV). Although the statistical significance for the risk of PTB in the *IL1A* −889C/T and +4845G/T polymorphisms was not preserved by multiple comparison tests using the Bonferroni correction, the statistical significance for the risk of PTB in the *IL1A* TT and CG haplotypes persisted through 10 000 permutation tests ($P = 0.01$ for both). These findings suggest that the *IL1A* TT haplotype may be a potential modifier for the risk of PTB, whereas the CG haplotype may be rather protective against PTB. On the other hand, the association between the *IL1A* polymorphisms and LBW seemed to be a secondary outcome dependent on PTB because its statistical significance was completely lost by the logistic analysis adjusted for gestational age (data not shown).

The present study has several limitations. First, our sample size was not large enough, especially among the subgroups of PTB, to examine possible associations with common proinflammatory cytokine polymorphisms in a Japanese population. However, it was sufficient to detect a significant risk for PTB with the *IL1A* polymorphisms. Second, the functional consequences of *IL1A* polymorphisms could not be examined in the present study; previous investigations, however, have suggested functional changes in subjects carrying those polymorphisms (Hulkkonen *et al.*, 2000; Dominici *et al.*, 2002; Li *et al.*, 2004). Third, we could only obtain partial information for the subjects regarding socio-economic status, maternal weight and height before and during pregnancy, previous histories of PTB, LBW, cervical procedures and interventions in our registration system. Therefore, we could not exclude these data as possible confounding factors in the statistical analyses. However, the selected confounding factors shown in Table I, which were often used in previous studies, did not affect our findings whatsoever. Fourth, our study population was comprised entirely of Japanese women. A previous report found that the *IL1A* +4845G/T polymorphism was modestly associated with spontaneous PTB in Caucasian women, whereas it had no association with spontaneous PTB in African American women (Engel *et al.*, 2005). Thus, the *IL1A* polymorphisms vary with ethnicity. Fifth, it was impossible to assess whether the variant in the *IL1A* gene might be the key or whether it is something they both tag in the present study because the two SNPs in *IL1A* were in high LD. Further studies with a larger population are needed to elucidate whether polymorphisms and haplotypes in *IL1A* and the *IL1* gene cluster are associated with which types of PTB in each ethnic group.

In conclusion, our findings suggest that polymorphisms and haplotypes in the *IL1A* gene are associated with PTB in Japanese women.

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REGULAR ARTICLE

Decreased maternal protein S activity is associated with fetal growth restriction

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KEYWORDS
Fetal growth restriction;
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Abstract

Introduction: Protein S (PS) activity has been shown to decrease during normal pregnancy. The aim of this study was to determine any correlation between decreased maternal PS activity and fetal growth restriction (FGR).
Methods: We carried out a retrospective study of maternal PS activity and complement 4b-binding protein (C4BP) concentration in 102 patients with FGR and 58 patients with fetuses that had normal growth. Among pregnancies affected by FGR, 14 diagnoses were made in the second trimester and 88 in the third trimester. Patients whose fetuses had normal growth were matched with FGR subjects for maternal age and gestational age at sampling (29 cases each in the second and third trimester).
Results: Mean PS activity of the control group in the third trimester was significantly lower than in the second trimester (56.5 ± 16.5% vs 35.8 ± 13.8%). PS activity in women with FGR was significantly decreased in both the second trimester (36.6 ± 13.2%) and third trimester (30.2 ± 12.2%) compared with control group levels. Plasma concentrations of C4BP for the control group were significantly higher in the third trimester than in the second trimester (90.5 ± 17.5% vs 81.1 ± 13.6%). However, in women with FGR, plasma C4BP concentrations in both the second trimester (84.0 ± 14.8%) and the third trimester (86.0 ± 17.7%) were comparable with concentrations of the control group.
Conclusions: Maternal PS activity decreased as normal pregnancies progressed but decreased over time in cases with FGR. Excessive decreases in PS activity during pregnancy could contribute to development of FGR.

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Introduction

Fetal growth restriction (FGR) is a common complication of pregnancy associated with a failure of normal placental invasion and development [1]. The consequences of this placental dysfunction carry a significant increased risk of fetal morbidity and mortality as well as possible later deficits in a child's neuropsychological development [2]. Recently, several studies have focused on a link between maternal inherited thrombophilia and FGR: Researchers found that the frequency of maternal inherited thrombophilia, which may be accompanied by deficiencies in factors such as antithrombin (AT), protein C (PC), or protein S (PS), was significantly increased in the FGR group of mothers compared with the control group [3–5]. In cases of maternal thrombophilia associated with FGR, maternal floor infarction of the placenta, which is characterized by deposition of fibrinoid material, could be found not only in the maternal surface but also in intervillous spaces of the placenta [6]. Thus, both maternal thrombophilia and infarction of intervillous spaces of the placenta could be causes of FGR.

On the other hand, it has been reported that PS activity showed a progressive decrease during pregnancy in women without inherited PS deficiency, and this condition is called "acquired PS deficiency" [7–9]. It has also been reported that the level of C4BP increases during pregnancy [10]; hence, an increased C4BP level may contribute to reduced PS activity during pregnancy. When protein S activity falls below 35%, clotting time is shortened, thus increasing the risk of developing venous thromboembolism [10]. However, it has not been revealed whether decreased PS activity during pregnancy could contribute to development of FGR. In the present study, we investigated maternal PS activity in patients who fetuses demonstrated FGR to determine if there was a correlation between FGR and decreased PS activity.

Materials and methods

Study population

We studied 102 subjects whose fetuses demonstrated FGR and 58 subjects whose fetuses had normal growth. All were followed at the Maternity and Perinatal Care Unit of Kyushu University Hospital from January 1997 to December 2004. The gestational ages were calculated from the date of the last menstrual period and confirmed sonographically between 9 and 11 weeks of gestation. The Institutional Ethics Committee approved the study, and all mothers gave informed consent prior to participation.

Fetal growth was considered normal when the birth weight was appropriate for the gestational age (within the mean ± 10th percentile for the gestational age). FGR was defined as having both estimated fetal body weight and birth weight lower than the 10th percentile for gestational age according to Japanese standards [11,12]. None of the cases in our analysis involved maternal or fetal complications such as intrauterine fetal death, fetal genetic aberrations, eclampsia, preeclampsia, abruptio placentae, gestational hypertension, preterm labor, preterm rupture of membranes, or other medical disorders. No mothers had a history of stroke or thromboembolic disease.

After verifying that fetuses were viable, we obtained blood samples from subjects with FGR at the time of diagnosis. Among FGR pregnancies, 14 were diagnosed in the second trimester (14–<28 weeks), and 88 were diagnosed in the third trimester (≧28 weeks). We also obtained a total of 58 blood samples from subjects whose fetuses had normal growth, and these were matched for maternal age and gestational age at sampling (29 cases, second trimester; 29 cases, third trimester).

Measurement variables

PS activity was determined by the coagulometric method (Staclot Protein S kit, Diagnostica Stago, Asnieres, France). Plasma concentrations of complement 4b-binding protein (C4BP) were determined by latex agglutination (Liatest C4b-BP kit, Diagnostica Stago, Asnieres, France). Normal values of PS activity in nonpregnant women were set between 59% and 128% [13]. When

Table 1 Clinical profiles of control and FGR subgroups throughout pregnancy

	Second trimester		Third trimester	
	Control (N=29)	FGR (N=14)	Control (N=29)	FGR (N=88)
Maternal age (years)	31.1±5.7	32.1±8.9	31.7±4.5	31.8±6.2
GA at sampling (weeks)	25.3±1.0	25.9±1.2	33.5±3.5	34.6±2.8
GA at delivery (weeks)	38.4±2.5	32.6±6.6*	38.8±2.1	37.0±2.3**
Birth weight (g)	3180±406	1370±577*	3022±5457	1955±491**
Platelet count ($\times 10^4/\mu l$)	22.7±7.4	23.8±7.7	22.0±5.9	22.8±6.7
PT-INR (%)	0.99±0.07	0.98±0.06	0.99±0.04	0.98±0.02
APTT (seconds)	30.9±4.03	30.5±4.14	31.1±3.63	30.9±3.27

FGR, fetal growth restriction; GA, gestational age; PT-INR, prothrombin time-international normalized ratio; APTT, activated partial thromboplastin time.

* In the second trimester, the mean gestational age at delivery and infant birth weight were significantly lower in FGR groups compared to the control groups (ANOVA, $P<0.003$).

** In the third trimester, the mean gestational age at delivery and infant birth weight were significantly lower in FGR groups compared to the control groups (ANOVA, $P<0.001$).

measurements of PS activity were below the lower limit of the normal range, we re-evaluated PS activity at 1 month postpartum, and inherited defects were considered when repeat tests showed values similar to the original measurements. In addition, subjects with a deficiency of AT or PC, or the presence of anticardiolipin antibodies and lupus anticoagulant, were excluded from the present study.

Statistical analysis

All data are presented as mean ± SD. Two-way analysis of variance (ANOVA) was used to analyze for differences in PS activity and C4BP among subgroups. If there were statistically significant differences, the Bonferroni method was applied for multiple comparisons. A P value of less than 0.05 was considered to be statistically significant; note that P values in text and figures are for tests done without the Bonferroni correction. Statistical analyses were performed with a statistical software package (StatView Version 5.0; SAS Institute, Cary, N.C., USA).

Results

Table 1 shows the maternal age, gestational age at blood draw, and platelet count and coagulation data in each trimester for the 58 women of the control group and the 102 women whose pregnancies showed FGR. For each trimester, there were no statistically significant differences between these two groups except for gestational age at delivery and infant birth weight (Table 1).

In the normal control group, mean PS activity in the third trimester (37.5 ± 11.5%) was significantly lower than levels measured in the second trimester (56.5 ± 16.5%; ANOVA, $P<0.0001$, 0.1% significance with Bonferroni correction) (Fig. 1). In the FGR group, PS activity did not decrease significantly between the second and third trimesters (ANOVA, second trimester 36.6 ± 13.2% vs. third trimester 30.2 ± 12.2%; $P=0.146$). In the comparison of PS activity during each trimester between control and FGR groups, PS activity

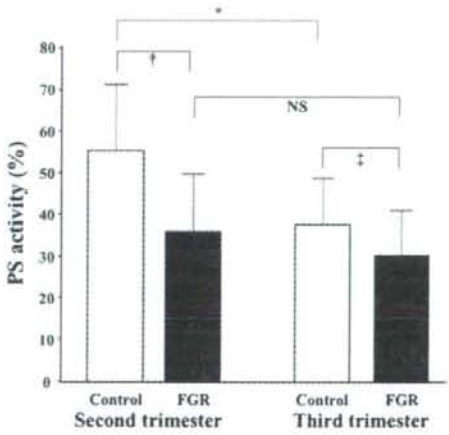

Figure 1 PS activity at the second and third trimesters in the control group and the FGR group. Open square, the control group; solid square, FGR group. †$p<0.0001$, 0.1% significance with Bonferroni correction. *$p<0.0001$; 0.1% significance with Bonferroni correction. ‡$p=0.010$; 5% significance with Bonferroni correction. NS, not significant.

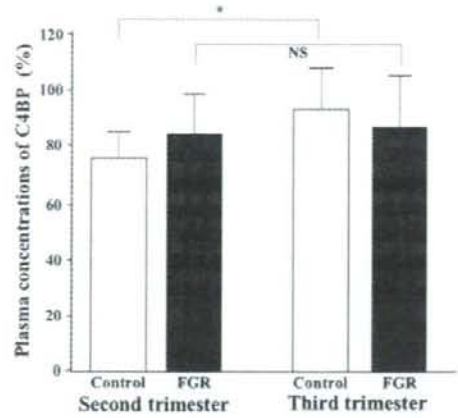

Figure 2 Concentrations of C4BP at the second and third trimesters in the control group and the FGR group. Open square, the control group; solid square, FGR group. C4BP, complement 4b-binding protein; FGR, fetal growth restriction. *$p=0.004$, 5% significance with Bonferroni correction. NS, not significant.

levels for the FGR group in both the second and third trimester were significantly decreased compared with levels in the control group (ANOVA, $P<0.0001$ and $P=0.010$, 0.1% and 5% significance with Bonferroni correction, respectively).

Plasma concentrations of C4BP for the control group in the third trimester were significantly higher than those measured in the second trimester (ANOVA, second trimester 75.5 ± 9.6% vs. third trimester 93.4 ± 14.0%; $P=0.004$, 5% significance with Bonferroni correction) (Fig. 2). In the FGR group, plasma C4BP concentrations did not increase significantly between the second and third trimesters (ANOVA, second trimester 84.0 ± 14.8% vs. third trimester 86.0 ± 17.7%; $P=0.759$). When plasma concentrations of C4BP during each trimester were compared between control and FGR groups, there were no significant differences in either the second or third trimester between groups (ANOVA, $P=0.283$ and $P=0.092$, respectively).

Two of 98 subjects (2.0%) with FGR had a confirmed PS deficiency after reevaluation of PS activity at 1 month postpartum, whereas none of the control group had a PS deficiency.

Discussion

PS is a vitamin K-dependent glycoprotein that is synthesized primarily in hepatocytes, endothelial cells and megakaryocytes [14]. About 40% of protein S is present as free PS, and it participates in both activated protein C (APC)-dependent and APC-independent mechanisms of anticoagulation, both of which result in down-regulation of thrombin and factor Xa generation. However, approximately 60% of PS forms a complex with C4BP, which has no anticoagulation function.

Inherited PS deficiency has been identified in 1–7.5% of patients with deep venous thromboembolism (DVT) and in 0.03–0.13% of the general

Caucasian population [15]. However, Japanese people have a higher prevalence of PS deficiency, both in DVT patients (12.7%) and in the general population (0.48–0.63%) [7]. In studies, the frequency of PS deficiency was significantly increased in FGR groups compared with control groups [16–18]. According to a review by Alfirevic et al. [16], women with FGR had associated PS deficiency more often than did controls, with a pooled odds ratio and 95% confidence interval (CI) of 10.2 and 1.1–91, respectively. In the present study, 2 of 98 subjects (2.0%) with FGR had a PS deficiency, whereas none of the control group members had a PS deficiency. These findings suggest that decreased maternal PS activity may lead to development of FGR.

Mean PS functional levels decline strikingly from the first to third trimester of pregnancy [19]. The present result, demonstrating that PS activity was significantly decreased from the second to third trimester in normal pregnancies, is consistent with previous reports [7–9]. On the other hand, it has been reported that the level of C4BP increases during pregnancy [10]. Our study showed that the C4BP level of subjects in the third trimester was significantly higher than that in the second trimester among women with normal pregnancies. This suggests that an increased C4BP level may reduce PS activity during pregnancy.

On the other hand, PS activity of the FGR group in this study was significantly lower than that of the control group in both the second and third trimesters. However, the plasma C4BP concentrations in each trimester did not differ between groups. This suggests that the decreased PS activity observed in women with FGR is not only associated with increased C4BP level but also with other factors. Patients with AT deficiency, PC deficiency, anticardiolipin antibodies, or lupus anticoagulant were excluded from this study. In addition, no included subject exhibited any thromboembolic symptoms, and family histories were examined carefully to exclude patients with thromboembolic tendencies. Our results indicate that FGR is associated with decreased maternal PS activity. Thus, decreased PS activity may contribute to the development of FGR. In this study, we could neither prove a causal relationship between decreased PS activity and development of FGR nor determine how the degree of decrease in PS activity during pregnancy may indicate a greater risk for FGR. Clarification of these matters requires further interventional study with larger groups; enrollment would have to precede development of FGR and also be based on the degree of decrease in PS activity.

The etiology and mechanisms underlying FGR are not clearly understood. It has been suggested that FGR is associated with abnormal placental vasculature and disturbances of hemostasis leading to inadequate maternal–fetal circulation [20,21]. Placental histological examination showed that patients with FGR had more lesions of uteroplacental insufficiency or chronic villitis than did placentas of patients with preterm infants that had grown appropriately [22]. Arias et al. found that thrombotic lesions were present in 7 of 13 placentas of patients who had an adverse pregnancy outcome together with evidence of a thrombophilic state in the mother [23]. Among subjects with low free protein S levels, three of eight placentas had placental infarction and three had intervillous thrombosis [24]. These findings suggest that a similar pathogenic mechanism might have caused FGR in our subjects with decreased PS activity. Whether abnormal clotting occurs in the fetal, maternal, or both placental circulations remains to be examined.

Because uteroplacental thrombosis is a feature of FGR in women with thrombophilia, prophylaxis with heparin has been offered as anticoagulant therapy in pregnancy to prevent adverse pregnancy outcomes including FGR in women with antiphospholipid syndrome or other thrombophilias [25]. If it is confirmed by further studies that vascular injury and thrombin generation due to decreased PS activity causes FGR in addition to inherited thrombophilia, such prophylactic antenatal anticoagulation for patients with decreased PS activity might be anticipated.

Finally, two notes are in order about our study design. We were limited to identifying appropriate matched pregnancies from the total number of normal pregnancies delivered at our institution during the study period. We were able to identify 29 second-trimester control cases compared with 14 FGR pregnancies, but only 29 third-trimester control cases compared with 88 third-trimester cases. A larger number of third-trimester control cases might well have strengthened our findings. The strength of our findings is also affected by our choice in statistical tests. We chose to do our primary analysis with two-way ANOVA, with adjustment during multiple comparisons with use of a Bonferroni correction. A peer suggested that this may have been too conservative and that use of either Neuman–Keuls or Scheffes testing would have been appropriate and have yielded stronger findings. We reviewed our decision-making and decided to remain with our chosen testing, but it is always worth noting that statements of statistical significant depend on the choices made in statistical testing.

In conclusion, FGR pregnancies that were diagnosed in both the second and third trimester had PS activity levels that were lower than those measured in normal pregnancies. This suggests that excessively

decreased PS activity during pregnancy could induce thromboses or infarctions of the placenta, and these could contribute to development of FGR.

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Immunisation with a plasmid DNA vaccine encoding gonadotrophin releasing hormone (GnRH-I) and T-helper epitopes in saline suppresses rodent fertility

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Summary Research into active immunisation against gonadotrophin releasing hormone (GnRH-I) has gained widespread acceptance as a means of controlling reproduction and behaviour of farm, companion and wild animals. Many studies describe the use of multiple copies of the self-peptide in linear alignment and conjugation with a large carrier protein to increase the immune response to the peptide. However, problems resulting from carrier protein epitope suppression have seen a diversion of interest into the use of genetic materials to elicit an optimum immune response. In this study, a 533-bp long DNA vaccine was constructed in pcDNAV5-HisB coding for 18.871 kDa GnRH-I-T-helper-V5 epitopes fusion protein. COS1 cells transfected with the vaccine construct were found to release fusion protein into culture supernatant. The vaccine construct (100 μg/mice) in saline solution administered into the anterior quadriceps muscle of ICR male and female mice stimulated antigen-specific IgG antibody responses. Testosterone levels in the vaccinated male mice were significantly ($p = 0.021$) reduced. A significant reduction in uterine implants were noted following mating between immunised males and control females ($p = 0.028$), as well as between immunised females and control males ($p = 0.004$). Histological examination of both the male and female gonads in study week 13 showed atrophy of the seminiferous epithelium and suppression of folliculogenesis.

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Introduction

Gonadotrophin releasing hormone (GnRH-I), also known as luteinizing hormone releasing hormone (LHRH), selectively stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary, which in turn regulates gonadal steroidogenesis and gametogenesis [1]. This function has attracted widespread attention to the significant potential of GnRH-I immunoneutralisation as a means of an anti-fertility target [2,3]. Moreover, expression of GnRH-I and its receptors in a number of malignant tumours of the breast, ovary, endometrium, and prostate in mammals [4] has also contributed to a significant interest in neutralizing GnRH-I to control tumour growth [5–7] and in other applications [8–11].

Although the induction of immune responses against GnRH-I peptide conjugates has proven efficacy in laboratory rodents, there are a number of setbacks in clinical and veterinary applications, which has resulted in few products on the market [12]. Self-peptide conjugates without adjuvant can exhibit low immunogenicity [9,12] and peptide conjugate preparations are highly heterogenous which makes them unsuitable for regulatory approval [11,13]. Both these problems have been partially obviated by administering GnRH-I immunogens in adjuvants [14] or by conjugating the GnRH-I peptide to defined T-helper epitopes [6,15]. However, this procedure is expensive and not effective with approved clinical/veterinary adjuvants. This study is therefore, designed with a genetic immunisation strategy to neutralise native GnRH-I. The plasmid DNA vaccine we used is a further improvement on our previous construct [16], which successfully induced anti-GnRH antibody responses in ICR male mice delivered in conjunction with a hemagglutinating virus of Japanese envelop (HVJE) vector and in saline solution. Since the encoded protein was not released into culture medium following transfection of COS1 cells using this particular construct [16], a V5 epitope from the feline immunodeficiency virus was tagged onto the C-terminal of the construct to help the fusion protein to be secreted following transfection and make it available for immune recognition. To overcome the disadvantages of using a viral vector/adjuvant system, the vaccine construct in saline solution was evaluated in male and female mice in terms of anti-GnRH-I antibody responses, serum testosterone levels, altered gonadal function and suppressed fertility *in vivo*.

Materials and methods

Engineering of the vaccine

In this study, the parent vaccine in pcDNA3.1+ plasmid was modified by slicing at Xba1 sites and a 440-bp long fragment was ligated into the mammalian expression vector pcDNAV5-HisB (Invitrogen Japan K.K., Tokyo, Japan) at Xba1 sites. The plasmid backbone of pcDNAV5-HisB contains a V5 epitope from the feline immunodeficiency virus and a histidine tag before the stop codon. A competent *Escherichia coli* strain DH5α (50 μl, 10^{7-8} CFU) was used to amplify 1 ng/1 μl plasmid DNA in LB medium containing 100 μg/ml ampicillin and amplified using endotoxin free plasmid mini and giga prep kits (Qiagen, Hilden, Germany). The T-7 primer of the vector (ttaatacgactcactataggg) and a reverse gene (acgatcacgcctttgatttc) sequence (Gene Design, Japan) obtained from the terminal sequence of the vaccine were used in a polymerase chain reaction (PCR) to select transformed bacterial colonies. The gene sequence of the vaccine in pcDNAV5-HisB was confirmed by a Big Dye Kit (PE-biosystem) in ABI PRISM 310 Genetic Analyzer using the T-7 primer and reverse gene sequence. The nucleotide sequence data and deduced amino acid sequence were analysed with DNASIS-Mac (v.3.2) computer software (Hitachi Software, Ibaraki, Japan).

Gene sequence of the vaccine

Underlined sequences indicate GnRH-I, italic letters theV5 epitope and solid triangles represent the Xba1 cutting and ligation sites.

t▼ct aga (start) atg aag cca att caa aaa ctc cta gct ggc ctt att cta ctg act tcg tgc gtg gaa ggc tgc tcc agc cag cac tgg tcc tat gga ctg cgc cct gga gga agc ggt gat atc gaa aaa aaa atc gcg aaa atg gaa aaa gcg agc agc gtg ttt aac gtg gtg aac ggt aag ctt agc gga gaa cat tgg agc tat ggc ctg cgt ccg ggc agc ggt gcg gaa tat aac gtg ttt cat aac aaa acc aaa gaa ctg ccg cgt gcg ggt ggt gaa cat tgg agc tat ggc ctg cgc ccg ggc ggt ggt cag tat atc aaa gcg aac agc aaa ttt atc ggc att acc gaa ctg ggt agc ggt gaa cat tgg agc tat ggc ctg cgt ccg ggc ggt agc ggt ctg agc gaa atc aaa ggc gtg atc gtg cat cgt▲ cta gag ggc ccg cgg ttc gaa *ggt aag cct atc cct aac cct ctc ctc ggt ctc gat tct acg* cgt acc ggt cat cat cac cat cac cat tga (stop).

Translated protein

Underlined sequences are GnRH-I, bold letters Th2 epitopes of circumsporozoite protein (CSP) of *Plasmodium falciparum*, respiratory syncitial virus (RSV), measles virus protein (MVP) and tetanus toxoid (TT), respectively and italic letters represent the V5 epitope.

MKPIQKLLAGLILLTSCVEGCSSQHWSYGLRPGGSG**DIEKKIAKMEKASSVFNVVNGKL**SGEHWSYGLRPG**SGAEYNVFHNKTFELPRA**GGEHWSYGLRPGGGQ**YIKANSKFIGITELGSGE**HWSYGLRPG GSG**LSEIKGVIVHRLEGPRFE***GKPIPNPLLGLDST***RTGHHHHHH-Stop

Gene design of the vaccine

Met---------GnRH-I leader sequence--Gly-Ser-Gly----------CSP----------Lys-Leu-

Ser-Gly----GnRH-I----------Ser-Gly----------RSV----------Gly-----

Gly---------GnRH-I----------Gly-Gly----------TT----------Gly-----

Ser-Gly----GnRH-I----------Gly-Ser-Gly----------Measles----------Pro-----

Arg-Pha-Glu---------V5-epitope----------Histidine-Tag----------Termination.

Western blotting detection of fusion protein

The fusion protein in Western blotting membrane was detected with COS1 cells lysate and culture supernatant transfected with 4 μg/well (6-well plates) of vaccine construct or naked plasmid in Lipofectamine™ 2000 [16]. 30 h after transfection, COS1 cell culture supernatant and cell lysate were collected in ice-cold lysis buffer containing 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.1% (w/v) sodium dodecyl sulphate (SDS), 1 mM dithiothreitol (DTT), 10 mM NaF, 2 mM Na_3VO_4, 1× phenylmethylsulphonyl fluoride (PMSF) and 1× complete protease inhibitor cocktail (Boehringer Mannheim, Ingelheim, Germany). Cell lysate was prepared by sonication and supernatant was collected by centrifuging at 2000 × *g* for 15 min at 4 °C. The protein concentration of the culture supernatant and cell lysate was determined by Bradford assay. Each soluble protein sample (30 μg) was separated by 15% SDS-PAGE and blocked in 5% (w/v) skimmed milk/TPBS (1× PBS pH 7.4, 0.1% (v/v) Tween-20). These samples were probed with 7 ml 1:1000 dilution of anti-V5 antibody (Invitrogen Life Technologies, Inc., Grand Island, NY) or GnRH-I specific monoclonal antibody (Santa Cruz, CA, USA, cat. no. sc-33675) and 7 ml horseradish peroxidase-conjugated goat anti-mouse IgG (1:3500 dilution, Santa Cruz, CA, USA, cat. no. sc-2005). The protein signals in the membrane were detected using an enhanced chemiluminescence (ECL) reagent (Pierce, USA). The translated fusion protein in the transfected rat muscle lysate was also used to detect fusion protein by Western blotting analysis.

Fluorescent detection of fusion protein in rat muscle

Western blotting analysis failed to detect fusion protein in the transfected rat muscle. In order to explore expression of fusion protein *in vivo* rat muscle was transfected with the vaccine construct and the translated fusion protein was detected using immunohistochemistry. In brief, 10-week-old Sprague–Dawley male rats were anaesthetised intraperitoneally (i.p.) with pentobarbital sodium (3.5–4 mg/100 g BW, Nembutal Injection, Dainippon Pharmaceutical Co. Ltd., Japan) and 200 μg vaccine construct in 100 μl saline solution was injected into the left anterior quadriceps muscle. Anterior quadriceps muscle from untreated male rats served as controls. 30 h after transfection, the rats were sacrificed by injecting with pentobarbital sodium (10 mg/100 g BW) and the anterior quadriceps muscle collected. 4 μm thick cryosections were fixed with 4% (v/v) paraformaldehyde in phosphate-buffered saline (PBS) for 30 min and then washed 3× (15 min) in distilled water. Non-specific binding sites on the muscle cells were saturated at room temperature with 10% (v/v) normal goat serum (DakoCytomation, Glostrup, Denmark). After 30 min, the serum was poured off and the sections were incubated overnight at 4 °C with 1:100 dilution of mouse monoclonal anti-V5 antibody (Invitrogen Life Technologies, Inc., Grand Island, NY). The anti-V5 antibody was allowed to react with a secondary antibody (goat anti-mouse IgG1; dilution, 1:50) labelled with fluorescein isothiocyanate (FITC, Santa Cruz Inc., CA, USA, cat. no. sc-2078) followed by a nuclear counterstaining with bisbenzimide trihydrochloride (Hoechst 33258) for 15 min to facilitate identification of morphologic features. Sections were mounted on glass slides in Dako fluorescent mounting medium (Dako) and observed under a confocal microscope (Olympus DP-70) equipped with Olympus U-MCB laser sources.

Immunisation of ICR mice

Three-week-old ICR male ($n = 10$) and female ($n = 10$) mice (SLC, Shizuoka, Japan) were housed in the Animal Care Services facilities at the Osaka University Graduate School of Medicine, Japan. The conditions were climate controlled to maintain ambient temperatures between 21 and 23 °C with a 14-h light:10-h dark cycle. Food and water were supplied at all times. Three days after caging, the mice were randomised, divided into groups of five (Groups 1–4) and ear coded. Mice were deeply anaesthetised with an i.p. injection of pentobarbital sodium (3.5–4 mg/100 g BW) and immunised in study weeks 0, 1, 2, 4 and 7. The vaccine construct, 100 μg was dissolved in 100 μl saline solution and were injected into the right and left anterior quadriceps muscles of Group 1 male and Group 3 female mice (50 μl/site). As negative controls, naked pcDNAV5-HisB plasmid, 100 μg was dissolved in 100 μl saline solution and 50 μl/site was injected into the right and left anterior quadriceps muscles of Group 2 male and Group 4 female mice.

Measurement of anti-GnRH-I antibody response

In study weeks 0, 3, 6, 9 and 12, 50–100 μl tail bleeds were carried out into heparinised capillary tubes (Fisher Scientific, Pittsburgh, PA). The plasma was prepared by centrifugation of the capillary tubes at 500 × *g* for 5 min and stored at −20 °C until a GnRH-I specific, end-point titre ELISA [16] was carried out on individual animal plasma, starting with a 1:100 dilution and 1:2 serial dilutions. The plate was developed with horse radish peroxidase (HRP) labelled rabbit anti-mouse IgG (1:2500 dilution, Acris Antibodies, Germany) and tetramethyl benzidine (TMB) substrate.

In vivo fertilization assay

Two mating trials for the males and one trial for the females were carried out. Vaccinated (Group 1) and control male mice (Group 2) were allowed to mate individually with 10–12-week old untreated virgin ICR female mice in study weeks 10 and 12. Similarly vaccinated (Group 3) and control female mice (Group 4) were mated individually with 12-week old untreated ICR male mice in study week 12. Female mice were examined every morning for vaginal plugs. Following 5 days of mating, the female mice were grouped in cages and feed and water were supplied *ad libitum*. After 2 weeks, the female mice were sacrificed by i.p. injection with 5 mg pentobarbital sodium to reveal the number of implants in the uterus.