Contraction-associated proteins expression by human uterine smooth muscle cells depends on maternal serum and progranulin associated with gestational weight gain

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Abstract. Pregnant women with obesity are at increased risk of parturition dysfunction; however, the biological mechanism has remained unknown. We hypothesized that molecules circulating in the serum of pregnant women with obesity may induce the aberrant expression of contraction-associated proteins (CAPs), leading to insufficient uterine contractions. This study aimed to investigate the effects of maternal serum on CAPs expression by human uterine smooth muscle cells (UtSMCs) and elucidate the influence of maternal obesity. Blood samples were collected from singleton pregnant women at 36–41 weeks of gestation before the onset of labor. UtSMCs were incubated in the serum, and the mRNA expressions of PTGFR, OXTR, GJA1, and PTGS2 were examined by RT-PCR. Progranulin (PGRN) is a circulating glycoprotein associated with insulin resistance characterized by the accumulation of visceral fat. The serum PGRN levels of the samples were measured by ELISA. After incubated with PGRN (100–1,000 ng/mL), mRNA expression of PTGFR, OXTR, and GJA1 and protein expression of CX43 were examined by RT-PCR and western blotting, respectively. The mRNA expressions of PTGFR, OXTR, and GJA1 showed significantly negative correlations with gestational weight gain (GWG). Serum PGRN levels showed a significantly positive correlation with GWG. High levels of PGRN suppressed the mRNA expression of GJA1 and the protein expression of CX43. The change in maternal serum induced by GWG suppressed the CAPs expression by UtSMCs. PGRN is one of the factors in the serum responsible for inhibiting the expression of CX43.

Key words: Gestational weight gain, Uterine smooth muscle cells, Contraction-associated proteins, Progranulin, Connexin 43

OBESITY, especially in women of reproductive age, has become an epidemic in many countries. The prevalence of obesity in pregnant women has increased over recent decades [1]. Women with obesity are at increased risk of delayed labor onset and slow labor progress, which often result in emergency cesarean delivery [2, 3]. However, the biological mechanism of this dysfunctional parturition associated with obesity is not well understood.

A strong and synchronized uterine contraction is essential for active labor. The coordinated expression of contraction-associated proteins (CAPs) by uterine smooth muscle cells (UtSMCs) is necessary for the effective myometrial contractions [4]. We hypothesized that molecules circulating in the serum of pregnant women with obesity may induce the aberrant expression of CAPs, as a result, leading to parturition dysfunction.

The current study was designed to investigate the effects of maternal serum on CAPs expression by UtSMCs and elucidate the influence of maternal obesity.

Progranulin (PGRN) is a secreted, 68.5-kDa glycoprotein. PGRN is a pleiotropic growth factor associated with many cellular processes including embryogenesis, neurodegeneration, (anti-)inflammation, wound repair, and lysosome function. Recent studies have revealed the importance of PGRN in promoting insulin resistance [5, 6], which suggests that PGRN may be one of the molecules responsible for the effects of maternal serum associated with obesity. We also investigated the association between serum PGRN levels and maternal obesity and subsequently examined the effects of PGRN on CAPs expressions by UtSMCs.

Besides, in discussions of ‘maternal obesity’ in pregnancy, it is unclear whether pre-pregnancy obesity or gestational weight gain (GWG) is more important. Our previous epidemiological study reported that GWG was an independent risk factor for delayed onset labor both in primiparous and multiparous women [3]. In this study, we also investigated the differential influence of pre-pregnancy obesity and excessive GWG on the CAPs expression by UtSMCs.
Material and Methods

Materials

Human uterine smooth muscle cells (UtSMCs) (Lot No. 6F0020) and the growth medium (Lot No. 828532) were purchased from Lonza (Basel, Switzerland). Human recombinant progranulin (PGRN) was purchased from R&D (Minneapolis, MN, US). Anti-CX43 antibody was purchased from Proteintech (Rosemont, IL, US).

Ethical approval and sample collection

Blood samples (5 mL) were obtained from singleton pregnant women at 36–41 weeks of gestation before the onset of labor at Kyorin University Hospital. All patients provided written informed consent for collection and research use of the blood samples. This study protocol was approved by the ethics committee of Kyorin University School of Medicine, Tokyo, Japan (IRB No. 1144). The blood sample was centrifuged at 1,500 × g for 10 min at room temperature. The serum was collected and stored at –80°C.

Cell culture

UtSMCs were seeded in flat-bottomed 12-well plates at 1.0 × 10^5 cells/well and cultured for 48 h in the growth medium at 5% CO₂ and 95% air at 37°C. After rinsing with PBS, the cells were incubated in the stored human serum or the serum-free medium with PGRN (0–1,000 ng/mL). After incubation for 24 h, the cells were collected for RT-PCR.

Semi-quantitative RT-PCR

Total RNA was extracted from the cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized by reverse transcription using the SuperScript™ III First-Strand Synthesis System (Thermo Fisher Scientific, Tokyo, Japan). Semi-quantitative RT-PCR was performed using QuantStudio 3 (Thermo Fisher Scientific) and Taqman® Fast Advanced Master Mix (Thermo Fisher Scientific). The primer pairs were purchased from Qiagen (PTGFR, Hs00168763_m1; OXTR, Hs00168573_m1; GJA1, Hs00748445_s1; PTGS2, Hs00153133_m1; GAPDH, Hs03929097_g1) and used according to the manufacturer’s instructions. For amplification, the cycling parameters consisted of 1 cycle at 50°C for 2 min and 95°C for 20 sec, followed by 40 cycles at 95°C for 1 sec and 60°C for 20 sec. The level of expression of each gene was calculated by the 2^-ΔΔCT method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene.

Immunoblotting

UtSMCs were seeded in the flat-bottomed 12-well plates at 1.0 × 10^5 cells/well and cultured for 48 h in the growth medium. After rinsing twice with PBS, UtSMCs were incubated in serum-free medium with PGRN (10–1,000 ng/mL) for an additional 24 h. After washing with ice-cold PBS, UtSMCs were solubilized with lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 2 mM EGTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 1 mM 2-[2-aminoethyl] benzenesulfonyl fluoride [AEBSF], 1 mg/mL pepstatin A, 1 mg/mL leupeptin, 1 mg/mL apro tinin). The insoluble materials were removed by centrifugation at 15,000 × g for 10 min at 4°C. The extracted proteins were eluted in 20 mL of Laemmli sample buffer, containing 200 mM dithiothreitol (DTT), and heated for 5 min at 95°C. The proteins were separated by SDS-PAGE on XX% polyacrylamide gels and transferred to PVDF membranes by electrophoresis. After blocking with Tris-buffered saline (10 mM Tris–HCl [pH 7.4] and 140 mM NaCl) containing 0.1% Tween-20 and 3% bovine serum albumin for 1 h at room temperature, the blots were exposed to anti-CX43 polyclonal antibody (1:1,000) overnight at 4°C. The blots were then incubated with secondary horseradish peroxidase-conjugated antibodies for 20 min at room temperature and visualized using enhanced chemiluminescence (ECL; Immobilon Western; Millipore). The obtained images were scanned using a LAS 4000 (GE Healthcare, UK). Densitometric analyzes of the images were performed using ImageJ (version 1.41, NIH, MD, USA).

Statistical analysis

The statistical analysis was performed using the SPSS Statistics version 21 software (IBM, Tokyo, Japan). All values from the cell experiments were expressed as the mean ± standard deviation (SD), and Student’s t-test was used to compare differences between the control and the test groups. The clinical data of the subjects were expressed as median (range). The correlation between values was assessed using Spearman’s correlation. A value of p < 0.05 was considered as statistically significant. The experiments for the effects of PGRN were repeated at least three times in each group to assess reproducibility.

Results

Effects of maternal obesity on expression of CAPs

To examine the effects of obesity on the expression of CAPs genes by UtSMCs, the cells were incubated in the serum of pregnant women with various degrees of obesity (N = 20). The demographic data of the included pregnant women are shown in Table 1. The relative mRNA expressions of the CAPs genes (PTGFR, OXTR,
GJA1 and PTGS2) by USMCs were plotted against pre-pregnancy body mass index (BMI) and GWG (Fig. 1). The relative mRNA expressions of PTGFR, OXTR, and GJA1 were significantly negatively correlated with GWG (PTGFR, $\rho = -0.686$, $p = 0.001$; OXTR, $\rho = -0.586$, $p = 0.007$; GJA1, $\rho = -0.528$, $p = 0.017$). None of these expressions showed a significant correlation with pre-pregnancy BMI (PTGFR, $\rho = 0.154$, $p = 0.516$; OXTR, $\rho = 0.005$, $p = 0.982$; GJA1, $\rho = -0.27$, $p = 0.245$). The expression of PTGS2 showed no significant correlation with pre-pregnancy BMI ($\rho = -0.105$, $p = 0.658$) or GWG ($\rho = 0.13$, $p = 0.596$). No distinctive distribution for gestational diabetes mellitus (GDM) patients was found in the plots.

**Effects of maternal obesity on serum progranulin concentration**

PRGN concentrations of the serum samples ($N = 38$) were measured by ELISA and plotted against pre-pregnancy BMI and GWG (Fig. 2A). The demographic data of the included pregnant women are shown in Table 2. The serum samples used in this experiment were different from those used for incubation with UtSMCs. Serum PRGN concentrations significantly were correlated with GWG ($\rho = 0.64$, $p < 0.001$) but not with pre-pregnancy BMI ($\rho = -0.18$, $p = 0.27$) (Fig. 2A). There was no significant difference in serum PRGN concentrations between GDM (394.5 ± 147.5 ng/mL) and normal glucose tolerance in pregnant women (368.5 ± 110.6 ng/mL) (Fig. 2B). Serum PRGN concentrations were not influenced by the gestational week of sample collection (37 weeks, 346.3 ± 149.3 ng/mL; 38 weeks, 352.7 ± 136.7 ng/mL; 39 weeks, 305.7 ± 125.6 ng/mL; 40 weeks, 447.0 ± 115.0 ng/mL; 41 weeks, 329.2 ± 123.4 ng/mL) (Fig. 2C).

**Table 1** Demographic data of the subjects for the experiments of UTSMCs incubation

|                        | $N = 20$                      |
|------------------------|------------------------------|
| Age at delivery (years)| 35.5 (24–45)                 |
| Primiparous            | 12/20                        |
| Gestational diabetes mellitus | 7/20                      |
| Pre-pregnancy body mass index (kg/m$^2$) | 21.8 (17.1–34.4)             |
| Gestational weight gain (kg) | 9.4 (–4.2–18.8)             |
| Gestational week of sample collection | 39 (36–41)                 |

**Fig. 1** The mRNA expression of CAPs genes and maternal obesity

The mRNA expressions of CAPs genes (PTGFR [A]; OXTR [B]; GJA1 [C]; PTGS2 [D]) by UtSMCs were examined by RT-PCR and normalized to GAPDH. The relative expression levels were scatter-plotted against pre-pregnancy BMI and gestational weight gain. Black dots represent gestational diabetes mellitus, and white dots represent normal glucose tolerance. $\rho$, Spearman’s rank correlation coefficient.
Effects of progranulin on expression of CAPs

The relative mRNA expressions of PTGFR, OXTR, and GJA1 by UtSMCs after incubation with PRGN were examined by RT-PCR (Fig. 3). Based on the results of PRGN ELISA, the concentrations of PRGN used for stimulation of UtSMCs were designated as 100, 500, and 1,000 ng/mL. PRGN did not affect the mRNA expression of PTGFR and OXTR. The mRNA expression of GJA1 was dose-dependently suppressed by PRGN. The protein expression of CX43 was examined by western blotting and found to be significantly decreased by 1,000 ng/mL of PRGN (p = 0.005 vs. control) (Fig. 4).

Discussion

The present study revealed that changes in maternal serum induced by GWG suppressed the CAPs expression by UtSMCs. The mRNA expressions of PTGFR, OXTR, and GJA1 were suppressed by incubation in the serum of the women with larger GWG (Fig. 1). Prostaglandin F receptor (FP), encoded by the PTGFR gene, and oxyto-
cin receptor (OTR) are the receptors for the contractile agonists prostaglandin F2α (PGF2α) and oxytocin, respectively. The expressions of FP and OTR in the myometrium increase from late gestation until delivery [7, 8].

Connexin 43 (CX43), encoded by the GJA1 gene, is a major gap junction protein in human myometrium. The upregulation of CX43 in the myometrium at labor onset and an increased number of gap junctions establish the ionic coupling among UtSMCs, which is essential for the synchronized contractions [9]. OTR antagonist, atosiban, and FP antagonist OBE0222 are known to suppress uterine contractions and induce uterine quiescence [10, 11]. Decreased expression of CX43 in human myometrium tissue in cases of prolonged labor was reported [12]. Based on this evidence, our results implicate that a decreased expression of CAPs can cause insufficient and irregular uterine contractions, which may explain the parturition dysfunction due to excessive weight gain.

These effects of the serum can be attributed to the changes in circulating molecules induced by weight gain during pregnancy.

Although pre-pregnancy obesity is also known to induce parturition dysfunction [2], pre-pregnancy BMI did not correlate with CAPs gene expressions in this study. This suggests a different mechanism is responsible for the parturition dysfunction due to pre-pregnancy obesity.

What is the difference in the pathophysiology between...
pre-pregnancy obesity and excessive weight gain? Obesity is characterized by the presence of an excessive amount of adipose tissue. The deposition of adipose tissue occurs in two different anatomic sites: visceral adipose tissue (VAT) and subcutaneous adipose tissue (SCAT). VAT is known to differ from SCAT in the endocrine function of adipokines and plays an important role in metabolic disorders such as increased insulin resistance [13]. It is known that VAT disposition is associated with a greater risk of diabetes and dyslipidemia [14]. Previous studies have investigated the longitudinal change in maternal adipose tissue deposition during pregnancy by ultrasonography [15, 16]. They revealed that adipose tissue distribution during pregnancy showed a tendency towards a decreased accumulation of SCAT and increased accumulation of VAT. On the other hand, non-pregnant young women tended to store adipose tissue in the subcutaneous sites as their weight increased [17, 18]. This evidence provides the speculation that excessive weight gain during pregnancy is more associated with the effects of VAT accumulation, whereas SCAT accumulation is predominantly responsible for pre-pregnancy obesity in young women. Therefore, it is hypothesized that molecules more associated with the accumulation of VAT, rather than SCAT, are responsible for our results.

Progranulin (PGRN), a 68.5-kDa glycoprotein, is associated with many cellular processes including the promotion of insulin resistance [5, 6]. Furthermore, PGRN is especially associated with the accumulation of VAT [19, 20]. Therefore, we turned our attention to PGRN and subsequently examined its role in the effects of the maternal serum associated with GWG.

The serum levels of PGRN in the pregnant women showed a significantly positive correlation with GWG but not with pre-pregnancy BMI (Fig. 2). An association of PGRN with BMI has been previously reported [6]; however, never with weight gain during pregnancy. To the best of our knowledge, we revealed, for the first time, a positive correlation between serum PGRN and GWG. The gestational weeks of the samples ranged from 37 weeks to 41 weeks, which did not influence the serum levels of PGRN. Although elevated PGRN has been reported in pregnancies with GDM [21], no associations were found in the current study. This discrepancy may be attributed to the relatively small size of the subject population.

Next, the effects of PGRN on the CAPs expression by UtSMCs were investigated. Incubation with a high concentration (1,000 ng/mL) of PGRN suppressed the expression of CX43 in both mRNA and protein levels. These results suggest that PGRN is one of the molecules responsible for the inhibition of uterine contractions due to GWG. Besides, the expressions of FP and OTR can be regulated by different molecules in maternal serum.

PGRN mediates its biological effects through tumor necrosis factor receptor 1 (TNFR1) [22]. Zang et al. reported that tumor necrosis factor-alpha attenuated the expressions of CX43 in spinal astrocytes [23, 24]. Those findings suggested that PGRN also inhibits the expression of CX43 via a TNFR1-dependent mechanism in UtSMCs, which should be examined in a future study.

In the present study, a functional assay of the contractile ability of UtSMCs was not performed. In addition, a functional assay of gap junctions is also necessary to show the significance of CX43 expression changes due to PGRN. Our results demonstrated only the potential roles of maternal serum and PGRN in parturition dysfunction due to excessive weight gain during pregnancy. Another limitation of this study was the relatively small sample size of the subject population for economic reasons because commercially purchased UtSMCs were used.

The samples were collected from the pregnant women before the onset of labor in order to exclude the possible influence of the active labor itself. Otherwise, we could not control for the phase of labor during serum sampling, which may have affected the results. Therefore, the UtSMCs incubated with the serum in the present study represented the pregnant uterus in the quiescence phase, rather than the active phase. The decreased expression of CAPs that depend on maternal weight gain may be implicated in delayed onset labor, rather than prolonged labor, which is consistent with our clinical evidence [3].

In conclusion, a change in maternal serum induced by GWG suppressed the CAPs expression by UtSMCs, possibly leading to parturition dysfunction. PGRN is one of the factors in serum responsible for inhibiting the expression of CX43. A comprehensive proteomics analysis of the maternal serum can help to select more candidate proteins for our results.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.
Reference

1. Ehrenberg HM, Dierker L, Milluzzi C, Mercer BM (2002) Prevalence of maternal obesity in an urban center. Am J Obstet Gynecol 187: 1189–1193.

2. Carlson NS, Hernandez TL, Hurt KJ (2015) Parturition dysfunction in obesity: time to target the pathology. Reprod Biol Endocrinol 13: 135.

3. Tanaka K, Muraoka Y, Honda R, Izawa T, Tanigaki S, et al. (2018) Significance of gestational weight gain in spontaneous onset of labor at term. J Obstet Gynaecol Res 44: 1915–1921.

4. Cook JL, Zaragoza DB, Sung DH, Olson DM (2000) Expression of myometrial activation and stimulation genes in a mouse model of preterm labor: myometrial activation, stimulation, and preterm labor. Endocrinology 141: 1718–1728.

5. Matsubara T, Mita A, Minami K, Hosooka T, Kitazawa S, et al. (2012) PGRN is a key adipokine mediating high fat diet-induced insulin resistance and obesity through IL-6 in adipose tissue. Cell Metab 15: 38–50.

6. Qu H, Deng H, Hu Z (2013) Plasma progranulin concentrations are increased in patients with type 2 diabetes and obesity and correlated with insulin resistance. Mediators Inflamm 2013: 360190.

7. Brodt-Eppley J, Myatt L (1982) Oxytocin receptors and human parturition: a dual role for oxytocin in the initiation of labor. Science 215: 1396–1398.

8. Garfield RE, Sims S, Daniel EE (1977) Gap junctions: their presence and necessity in myometrial parturition. Science 198: 958–960.

9. Goodwin TM, Paul R, Silver H, Spellacy W, Parsons M, et al. (1994) The effect of the oxytocin antagonist atosiban on preterm uterine activity in the human. Am J Obstet Gynecol 170: 474–478.

10. Pohl O, Chollet A, Kim SH, Riaposova L, Spézia F, et al. (2018) OBE022, an oral and selective prostaglandin F2α receptor antagonist as an effective and safe modality for the treatment of preterm labor. J Pharmacol Exp Ther 366: 349–364.

11. Cluff AH, Byström B, Klimaviciute A, Dahlqvist C, Cebers G, et al. (2004) Prolonged labour associated with lower expression of syndecan 3 and connexin 43 in human uterine tissue. Reprod Biol Endocrinol 4: 24.

12. Fontana L, Eagon JC, Trujillo ME, Scherer PE, Klein S (2007) Visceral fat adipokine secretion is associated with systemic inflammation in obese humans. Diabetes 56: 1010–1013.

13. Selovic A, Sarac J, Missoni S (2016) Changes in adipose tissue distribution during pregnancy estimated by ultrasonography. J Matern Fetal Neonatal Med 29: 2131–2137.

14. Enzi G, Gasparo M, Biondetti PR, Fiore D, Semisa M, Zurlo F (1986) Subcutaneous and visceral fat distribution according to sex, age, and overweight, evaluated by computed tomography. Am J Clin Nutr 44: 739–746.

15. Straughen JK, Trudeau S, Misra VK (2013) Changes in adipose tissue distribution during pregnancy estimated by ultrasonography. Mediators Inflamm 2013: 360190.

16. Youn BS, Bang SI, Klöting N, Park JW, Lee N, et al. (2009) Serum progranulin concentrations may be associated with macrophage infiltration into omental adipose tissue. Diabetes 58: 627–636.

17. Tanaka Y, Takahashi T, Tamori Y (2014) Circulating progranulin level is associated with visceral fat and elevated liver enzymes: significance of serum progranulin as a useful marker for liver dysfunction. Endocr J 61: 1191–1196.

18. Todoric J, Handsurya A, Perkmann T, Knapp B, Wagner O, et al. (2012) Circulating progranulin levels in women with gestational diabetes mellitus and healthy controls during and after pregnancy. Eur J Endocrinol 167: 561–567.

19. Zhou B, Li H, Liu J, Xu L, Guo Q, et al. (2015) Progranulin induces adipose insulin resistance and autophagic imbalance via TNFR1 in mice. J Mol Endocrinol 55: 231–243.

20. Zhang FF, Morioka N, Nakashima-Hisaoka K, Nakata Y (2013) Spinal astrocytes stimulated by tumor necrosis factor-α and/or interferon-γ attenuate connexin 43-gap junction via c-jun terminal kinase activity. J Neurosci Res 91: 745–756.

21. Zhang FF, Morioka N, Kitamura T, Hisaoka-Nakashima K, Nakata Y (2015) Proinflammatory cytokines down-regulate connexin 43-gap junctions via the ubiquitin-proteasome system in rat spinal astrocytes. Biochem Biophys Res Commun 464: 1202–1208.