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Myostatin in the placentae of pregnancies complicated with gestational diabetes mellitus

H.N. Peiris [a], M. Lappas [b, c], H.M. Georgiou [b], K. Vaswani [a], C. Salomon [a], G.E. Rice [a], M.D. Mitchell [a, *]

[a] The University of Queensland Centre for Clinical Research, Brisbane, Australia
[b] Department of Obstetrics & Gynaecology, The University of Melbourne, Mercy Hospital, Heidelberg, 3084, Victoria, Australia
[c] Obstetrics, Nutrition and Endocrinology Group, Department of Obstetrics and Gynaecology, University of Melbourne, Victoria, Australia

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ABSTRACT

Introduction: Gestational diabetes mellitus (GDM) is characterised by maternal glucose intolerance and insulin resistance during pregnancy. Myostatin, initially identified as a negative regulator of muscle development may also function in the regulation of placental development and glucose uptake. Myostatin expression in placentae of GDM complicated pregnancies is unknown. However, higher myostatin levels occur in placentae of pregnancies complicated with preeclampsia. We hypothesise that myostatin will be differentially expressed in GDM complicated pregnancies.
Methods: Myostatin concentrations (ELISA) were evaluated in plasma of presymptomatic women who later developed GDM and compared to plasma of normal glucose tolerant (NGT) women. Furthermore, myostatin protein expression (Western blot) was studied in placentae of pregnant women with GDM (treated with diet or insulin) compared to placentae of NGT women.
Results: No significant difference in myostatin concentration was seen in plasma of pre-symptomatic GDM women compared to NGT women. In placenta significant differences in myostatin protein expressions (higher precursor; $p < 0.05$and lower dimer: $p < 0.005$) were observed in GDM complicated compared to NGT pregnancies. Furthermore, placentae of GDM women treated with insulin compared to diet have higher dimer ($p < 0.005$) and lower precursor ($p < 0.05$). Compared to lean women, placentae of obese NGT women were lower in myostatin dimer expression ($p < 0.05$).
Discussion: Myostatin expression in placental tissue is altered under stress conditions (e.g. obesity and abnormal glucose metabolism) found in pregnancies complicated with GDM. We hypothesise that myostatin is active in these placentae and could affect glucose homoeostasis and/or cytokine production thereby altering the function of the placenta.

1. Introduction

Gestational diabetes mellitus (GDM) is characterised by maternal glucose intolerance leading to hyperglycaemia, β-cell dysfunction and insulin resistance [1]. GDM affects ~5% of all pregnancies globally [1–3]. GDM is associated with an increased short and long term risk of adverse outcomes for mother and infant, including a higher risk of induced labour, Caesarean section delivery, preterm birth, hypertension, preeclampsia stillbirth, macrosomia and infant respiratory distress syndrome, developing metabolic disorders, diabetes and cardiovascular disease [1,4–7].

Myostatin is a member of the Transforming Growth Factor-Beta (TGF-β) super family and members of this family function in the development of the placenta [8,9]. Comprehensive reviews are available on myostatin biosynthesis, signalling and function [8,10,11]. Myostatin is initially synthesised as a ~52 kDa precursor protein. Two proteolytic cleavages of the precursor release a ~42 kDa N-terminal pro-peptide and a ~12 kDa mature myostatin protein. The active form of myostatin is a homodimer of the mature protein. Myostatin circulating in blood is found mainly in a latent complex [10,12–14] composed of the active dimer bound non-covalently to two monomeric pro-peptides [10,13,15]. As a

* Corresponding author. UQ Centre for Clinical Research, Building 71/918, Royal Brisbane and Women's Hospital, Herston, 4029, QLD, Australia. Tel.: +61 7 33465500; fax: +61 7 3346 5509.
E-mail address: murray.mitchell@uq.edu.au (M.D. Mitchell).

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secreted protein in the human circulation [16], myostatin is thought to function in both an autocrine and paracrine manner [8,17].

Myostatin is best known as a negative regulator of muscle development [8]. Increased muscle, decreased adipose tissues and an improved metabolic status were observed in myostatin knockout mice (suppression of hyperglycaemia) and in mice with a loss of function mutation(s) to the myostatin gene (less insulin resistance) [8,18,19]. Specific inhibition of myostatin in muscle has been identified to be most effective in improving glucose metabolism and insulin sensitivity [20].

Expression of myostatin in the human placenta has been identified to be negatively correlated with gestational age, as lower protein levels in term compared to preterm human placentae [21]. Myostatin is localised to cytotrophoblast, syncytiotrophoblast and extravillous trophoblast cells of first trimester and term human placentae [14,22]. Following myostatin treatment alterations to glucose uptake of placental explants and BeWo cell (placental cell line) [21,23], and increased proliferations of primary isolated extravillous trophoblast cells [22] have been observed.

In complicated pregnancies, myostatin is known to be higher (mRNA and protein) in placentae of pregnancies complicated with preeclampsia [24]. A study by Hu et al. of serum myostatin concentrations of women with overt GDM found no significant difference [25]. However, no reports are available on the expression of myostatin in placentae of GDM. We hypothesise that myostatin will be differentially expressed in GDM complicated pregnancies. The current observational study evaluated myostatin expression in plasma of pre-symptomatic women who later developed GDM, to ascertain whether a difference in myostatin concentration can be seen earlier in pregnancy. Moreover, myostatin protein expression was evaluated in placentae of women with GDM and/or obesity, as myostatin expression in placentae GDM complicated pregnancies is currently unknown.

2. Methods

2.1. Ethics and collections (plasma and placentae)

Maternal blood was collected from pregnant women attending their first ante-natal visit (8–17 weeks gestation) at the Mercy Hospital for Women (MHW), Victoria Australia. Informed, written consent was obtained from participants (Mercy Health Ethics Committee, R08/31). After ultrasound confirmed a viable fetus and gestational age was established, 10 mL of venous blood was collected into an EDTA vacuum tube. The blood was centrifuged at 1000× *g* for 5 min, the plasma aliquoted into 1 mL microfuge tubes and immediately stored at −80 °C until required. After birthing, women with GDM ($n = 18$) and normal glucose tolerance (NGT, $n = 23$), were retrospectively selected for the study. GDM complicated pregnancies were not complicated with polycystic ovarian syndrome (PCOS), PE or IUGR. An a priori power analysis was performed using G*Power software [26]. Effect size of 0.5, in a two tailed student *t*-test, alpha error of 0.05 and power 0.3. The sample size required identified was $n = 18$ per group. Clinical and demographic details of participants are given in Table 1 (Mean ± SD).

Table 1
Clinical characteristics: myostatin in pre-symptomatic plasma.

	NGT ($n = 23$)	GDM ($n = 18$)
Maternal age (years)	31.98 ± 5.6	32.55 ± 4.6
Maternal BMI at sampling (kg/m^2)	26.7 ± 5.4	29.96 ± 6.9
Gestational age at sampling (weeks)	14.67 ± 1.8	13.19 ± 2.5
Gestational age at birth (weeks)	40.1 ± 1.2[‡]	39.15 ± 0.8
Fetal birth weight (kg)	3.4 ± 0.3	3.3 ± 0.3
Fasting plasma glucose (mmol/l)	4.33 ± 0.4[§]	5.17 ± 0.7
1 h plasma glucose (mmol/l)	6.91 ± 1.5[§]	10.88 ± 1.5
2 h plasma glucose (mmol/l)	5.97 ± 1.2[§]	10.46 ± 1.2

Values presented as mean ± SD.
[‡]$p = 0.0080$, [§]$p < 0.0001$ vs. GDM (one way ANOVA).

Mercy Health approved this study and written informed consent was obtained from participating women. Women with GDM were diagnosed according the Australasian Diabetes in Pregnancy Society (ADIPS) criteria by either fasting venous plasma glucose concentrations of ≥5.5 mmol/l glucose, and/or ≥8.0 mmol/l glucose 2 h after a 75 g oral glucose load at approximately 28 weeks gestation. Placenta was collected from women scheduled for an elective Caesarean section (with singleton pregnancies) at term (≥37 weeks gestation). Indications for Caesarean section were breech presentation and/or previous Caesarean section. Women with chorioamnionitis, preeclampsia, pre-existing diabetes, asthma, multifetal pregnancy, PCOS and fetal chromosomal abnormality were excluded. Placentae of women with NGT were collected as controls ($n = 22$ patients). Placentae of women with GDM were separated to those managed by diet alone ($n = 14$ patients) or insulin ($n = 20$ patients). Women were controlled by diet if their fasting glucose readings were maintained <5.5 mmol/l over a 2 week period post diagnosis. Women with fasting glucose readings >5.5 mmol/l were placed on insulin for optimal glucose control. Women were also stratified according to their pre-pregnancy BMI. Lean women were categorised as having a BMI ≤ 24.9 kg/m^2, overweight patients with a BMI ≥ 25 and ≤29.9, and obese patients with a BMI ≥ 30 kg/m^2. Clinical and demographic details of participants are given in Table 2.

Within 15 min of delivery placental cotyledons were obtained from various locations of the placenta; the basal plate and chorionic surface were removed from the cotyledon, and villous tissue was obtained from the middle cross-section. Tissues were blunt dissected to remove visible connective tissue and calcium deposits. Tissues were washed with PBS, immediately snap frozen in liquid nitrogen and stored at −80 °C until required.

2.2. ELISA

Myostatin ELISA was performed following the manufacturer's instructions (Immundiagnostik, Germany). A microtiter plate reader at 450 nm against 620 nm as a reference was used to read samples. A 4-parameter-algorithm was used to calculate the standard curve from which the concentration of myostatin was determined. The coefficient of variance between replicates was consistently below 10%. The detection limit of the kit is 0.273 ng/mL with a linear range 0–65 ng/mL. Controls tested (included in the kits) were within acceptable limits (C1 at 2–13 ng/mL and C2 at 15–29 ng/mL).

2.3. Western blot

Placental and ovine tissue (positive control) were extracted and Western blot performed (80 μg) according to previously described methods [27]. Membranes were blocked for 1 h in blocking solution (1 g non-fat milk powder and 1 g of Bovine serum albumin powder in 100 mL of Tris Buffered Saline with 0.001% Tween 20, Sigma Aldrich, Australia). Membranes were incubated overnight at 4 °C in myostatin primary antibody (Sc-34781 – Santa Cruz Biotechnology), at 200 ng/mL and secondary antibody at 20 ng/mL (Sc-2020 Santa Cruz Biotechnology) for 2 h. Bio-Rad Clarity western ECL substrate (Bio-Rad Laboratories Pty., Ltd., Australia) was used to visualise the proteins on Agfa medical X-ray blue film and developed using a Konica Minolta SRX-101A processor (Konica Minolta medical and graphic INC, Japan). The film was imaged on GS 800 calibrated densitometer, using Quantity One software (Bio-Rad