

The effect of treatment with the placental variant of human growth hormone during pregnancy on maternal and offspring outcomes in C57BL/6J mice

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Abstract

The human placental growth hormone variant (GH-V) is secreted continuously from the syncytiotrophoblast layer of the placenta during pregnancy, and is thought to play a key role in the maternal adaptation to pregnancy. Maternal GH-V concentrations are closely related to fetal growth in humans. GH-V has also been proposed as a potential candidate to mediate insulin resistance observed later in pregnancy. To determine the effect of maternal GH-V administration on maternal and fetal growth and metabolic outcomes during pregnancy, we examined the dose response relationship for GH-V administration in a mouse model of normal pregnancy. Pregnant C57BL/6J mice were randomized to receive vehicle or GH-V (0.25, 1, 2, 5 mg/kg per day) by osmotic pump from gestational days 12.5-18.5. Fetal linear growth was slightly reduced in the 5 mg/kg dose compared to vehicle and the 0.25 mg/kg groups respectively, whereas placental weight was not affected. GH-V treatment did not affect maternal body weights or food intake. However, treatment with 5 mg/kg per day significantly increased maternal fasting plasma insulin concentration with impaired insulin sensitivity observed at day 18.5 as assessed by HOMA. At 5mg/kg per day, there was also an increase in maternal hepatic GH receptor (*Ghr*) expression, but GH-V did not alter maternal plasma IGF-1 concentration or hepatic *Igf-1* mRNA expression. Our findings suggest that GH-V treatment causes hyperinsulinemia and is a likely mediator of the insulin resistance associated with late pregnancy.

Introduction

The growth hormone (GH) and insulin-like growth factor-1 (IGF-1) axis is a major regulator of mammalian growth. The human GH gene family, localised on chromosome 17p21, is a cluster of five tandemly arranged and highly related genes (1). Two GH genes encode two 22 kDa GH proteins: pituitary GH (GH-N; GH1) and placental GH variant (GH-V; GH2). The protein sequences of GH-N and GH-V are highly conserved, differing by 13 out of 191 amino acids (2) but they have distinct expression profiles; GH-N is predominantly secreted in a pulsatile fashion from the pituitary, while GH-V is secreted from the placenta in a nonpulsatile manner. The continuous secretion of GH-V into the maternal compartment is thought to contribute to maternal metabolic alterations during pregnancy (3). Both proteins bind the GH receptor (GHR) with similar affinity and share similar physiological somatotrophic, lactogenic and lipolytic properties (4, 5). However, GH-V binds the prolactin (PRL) receptor poorly and its lactogenic effects are greatly reduced compared with GH-N (6, 7). Following interaction with the GHR, GH stimulates the production and secretion of hepatic IGF-1, through activation of the JAK-STAT signalling pathway.

During pregnancy, concentrations of GH-N in the maternal circulation decline, whilst GH-V expression increases from week five, gradually replacing GH-N completely at approximately 20 weeks (3). The increase in maternal circulating GH-V is positively associated with fetal growth and circulating IGF-1 concentrations during pregnancy (8-12). A growth-promoting effect for GH-V has been demonstrated *in vivo* in non-pregnant hypophysectomized rats treated with GH-V and transgenic mice (7, 13, 14). Moreover, GH-V regulates placental angiogenesis and trophoblast invasion *in vitro* and may therefore play a role in the process of placentation (15, 16).

One of the characteristic features of the maternal adaptation to pregnancy is insulin resistance with resultant hyperinsulinemia (17). This environment ensures adequate nutrient supply to the fetus. However, increased insulin resistance can lead to gestational diabetes. Placental hormones, and to a lesser extent increased fat deposition during pregnancy, may contribute to insulin resistance during pregnancy (18, 19). Consistent with this, higher concentrations of circulating GH-V have been observed in pregnancies complicated by diabetes (9, 20). Furthermore, GH-V has been demonstrated to induce severe insulin resistance and alter body composition in non-pregnant transgenic mice that overexpress GH-V (14). Despite a proposed role for GH-V during pregnancy, the effects of GH-V administration on metabolic parameters and outcomes related to maternal and fetal growth are poorly understood.

In this study, we investigated the effect of GH-V on human and mice cell lines, and examined the dose response relationship for GH-V administration in a mouse model of normal pregnancy.

Materials and Methods

Cell lines and materials

The human prostate carcinoma cell line, LNCaP, and mouse myoblast cell line, C2C12, were obtained from the American Type Culture Collection (ATCC). LNCaP cells have previously been demonstrated to only express very low levels of *PRL receptor* mRNA (21). C2C12 cells express both *Ghr* and *Prl receptor* mRNA (22, 23). Cells were cultured at 37°C, 5% CO₂ in

RPMI (Gibco) supplemented with 10% heat-inactivated FBS, 100U/ml penicillin, 100µg/ml streptomycin and Glutamax (Gibco).

Recombinant human GH-V (22 kDa) was purchased from Protein Laboratories Rehovot (Rehovot, Israel) and was reconstituted in 0.4% NaHCO₃ pH 9 (24). Recombinant human GH-N (22 kDa) was obtained from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA, US).

Animals

All protocols were approved by the Animal Ethics Committee of the University of Auckland. Female C57BL/6J (B6) mice aged 8-10 weeks (Jackson Laboratories) were housed under standard conditions and maintained at 22°C with a 12 hours light/dark cycle and with ad-libitum access to food and water. Females were mated nightly with males and the day a vaginal plug detected was designated Gestational Day (GD) 0.5. Maternal body weight and food intake were monitored daily. At GD 12.5, pregnant mice were randomized to receive GH-V (0.25, 1, 2, or 5 mg/kg per day; calculated on the basis of maternal body weight at GD 11.5) or vehicle for six days by osmotic pump (Alzet model 1007D, Durect Corporation, Cupertino, CA) inserted on the animals back, slightly posterior to the scapulae. Maternal blood was obtained via tail tip at GD 12.5 and 15.5. At GD 18.5, pregnant mice were fasted for 6 hours, and euthanized by cervical dislocation; blood was collected by cardiac puncture. Glucose measurements were performed with a Freestyle Optium glucometer (Abbott, UK).

Maternal tissues, fetal and placental measurement

Maternal tissues, pups and placentas were dissected following euthanasia. Embryonic death was determined by the presence of fetal resorption, which appeared as dark round masses between live fetuses. Embryo resorption rate was calculated as number of reabsorbed embryos/total number of embryos of each group. Maternal liver, kidneys, spleen, pancreas, perirenal fat, retroperitoneal fat and gonadal fat weights, pup weights and placenta weights were recorded. Fetal crown-to rump lengths and abdominal circumferences were measured.

Plasma analysis

Plasma IGF-1 (Mediagnost, Germany) and insulin (CrystalChem, USA) were assayed with a mouse-specific enzyme-linked immunosorbent assay (ELISA) as per the manufacturers' instructions. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as: Fasting glucose (mmol/l)×fasting insulin (mU/l)/22.5 (25).

Quantitative real-time PCR

Total RNA was isolated from liver samples using Trizol (Life Technologies). The quantity and integrity of RNA were determined using a NanoDrop spectrophotometer (NanoDrop Technologies) and an Agilent Bioanalyzer RNA 6000 Nano kit, respectively. RIN numbers ranged from 7.6 to 8.4. Isolated RNA was DNase treated (Life Technologies). Single-stranded cDNA was synthesized from 1µg of RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche), according to the manufacturer's protocol. Real-time PCR analysis was carried out using predesigned PrimeTime qPCR assays (Integrated DNA Technologies) on a Lightcycler 480 (Roche). mRNA levels were normalized to 3 housekeeping genes: *Gapdh*, *β-Actin* and *Cox4i1* by subtracting the geometric mean Ct of housekeeping genes from the Ct for the gene of interest to produce a ΔCt value. The ΔCt for each treatment sample was compared with the mean ΔCt for vehicle-treated samples using the relative quantification 2-(ΔΔCt) method to determine fold-change (26).

Western blotting

Cells were grown to 70% to 80% confluence, serum starved for 16h and treated with 500nM GH-N or GH-V for 10 mins, prior to lysis in 50mM Tris-HCL pH 7.4, 1% Nonidet P-40; 150mM NaCl, 1mM EDTA, 1mM NaF, 1mM PMSF, 1mM Na3VO4, cOmplete protease inhibitor tablet (Roche) and sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). Where indicated, cells were treated with the human GHR antagonist, B2036 (500nM), for 30 min, prior to GH-V or GH-N treatment. Western blot analysis was carried out under reducing conditions using phospho-STAT5 (pTyr694) antibody (Life Technologies) or mouse β -ACTIN monoclonal antibody (Sigma-Aldrich). Proteins were visualized using horseradish peroxidase–conjugated secondary antibody with enhanced chemiluminescence on a BioRad Chemidoc MP system.

Statistical analysis

All normally distributed data are expressed as means \pm S.E.M and were compared using Student's *t* test or one way ANOVA with post-hoc analysis (Tukey's procedure or linear trend test) as appropriate. Maternal body weight and food intake data were analysed by repeated measures ANOVA. ANOVA analysis and regression analysis were conducted using SigmaPlot 12.0 and IBM SPSS Statistics 21, respectively. Linear and quadratic comparisons were made among doses. A p-value of <0.05 was accepted as statistically significant.

Results

Activation of the mouse GHR by GH-V

To confirm activity of the recombinant human GH-V used in this study, against the human and mouse GH receptor, activation of STAT5 signal transduction was determined in human and mouse cell lines by Western blotting. Both GH-N and GH-V stimulated STAT5 phosphorylation in the human prostate cancer cell line, LNCaP (Figure 1A), and the mouse myoblast cell line, C2C12 (Figure 1B). To determine whether GH-V activation of STAT5 occurred through binding to the GH receptor, we investigated *PRL receptor* expression. We were unable to detect *PRL receptor* expression in LNCaP cells by semi-quantitative RT-PCR (Supplementary Fig. 1). Furthermore, induction of STAT5 phosphorylation by GH-N and GH-V was abrogated by the specific GHR antagonist, B2036, thus confirming that phosphorylation of STAT5 was via activation of the GHR (Figure 1A and B).

Activation of the mouse GHR by recombinant human GH-V was confirmed in the mouse myoblast cell line, C2C12. *Ghr* and *Prl receptor* expression was detectable in C2C12 cells by semi-quantitative RT-PCR (Supplementary Fig. 1). Treatment with either GH-V or GH-N stimulated STAT5 phosphorylation in C2C12 cells (Figure 1B and C). B2036 treatment did not completely abrogate STAT5 activation by either GH-V or GH-N, indicating that GH-V and GH-N activate both the mouse GHR and PRL receptors in this cell line.

Maternal body weight and food intake

There was no statistically significant difference in maternal body weight at the time of mating or before osmotic pump implantation. Maternal body weight increased markedly with increasing gestational age in all groups (Figure 2A). However, there was no statistically significant difference in maternal body weight and food intake between the vehicle control and GH-V treatment groups (Figure 2A and B). A transient reduction in maternal food intake was seen in each group following osmotic pump implantation (Figure 2B).

Fetal growth and placental weight

There was no statistically significant difference in average litter size in each group (Table 1). Pup weight, fetal-abdominal circumference, and placental weight, as well as fetal/placental ratio were not significantly different at GD 18.5 (Figure 3A, B and D and Table 1). Interestingly, fetal crown-to rump length was reduced in the 5 mg/kg GH-V treatment group, when compared with the vehicle and 0.25 mg/kg treatment groups (29.51 ± 0.15 vs 28.73 ± 0.21 , $p < 0.05$ and 29.52 ± 0.13 vs 28.73 ± 0.21 , $p < 0.05$, respectively) (Figure 3C). Embryonic mortality was not changed by GH-V treatment, although a small increase in embryo resorption rate (6.56%) was observed in the 5 mg/kg GH-V treatment group (Table 1).

Maternal tissue weights

GH-V treatment did not affect the weights of maternal liver, kidneys, spleen or pancreas (Table 1). There were no significant differences in maternal adipose tissue weights across all treatment groups; however, we observed a significant dose effect of GH-V on perirenal fat weight (linear, $p < 0.05$; quadratic, $p < 0.05$), with an increase in perirenal fat weight associated with increasing GH-V dose (Figure 4A). A similar significant association with dose was observed on gonadal fat weights (linear, not significant; quadratic, $p < 0.05$) (Figure 4C). These results suggest that increased GH-V during pregnancy is associated with an increase in maternal adipose deposition.

IGF-1, fasting glucose and insulin levels

Maternal IGF-1 increased during mid-pregnancy and decreased in late pregnancy in all treatment groups (Table 1). However, GH-V treatment did not affect maternal IGF-1 plasma concentrations at either GD 15.5 or 18.5 (Table 1). Maternal fasting insulin levels were significantly increased and insulin sensitivity decreased in the 5 mg/kg treatment group at GD 18.5 (Figure 5A and B). A dose-dependent decrease in insulin sensitivity was observed (linear, $p < 0.01$; quadratic, $p < 0.05$) (Figure 5C). No affect was seen on fasting glucose levels (Table 1).

Hepatic mRNA expression

The effect of GH-V on hepatic mRNA expression was analysed by comparing gene expression in the vehicle-treated and 5 mg/kg GH-V treatment group (Figure 6). Hepatic *Ghr* expression was significantly up-regulated in the 5 mg/kg treatment group (1.30 ± 0.16 vs 1.99 ± 0.11 , $p < 0.01$). Solute carrier family 2, member 4 (*Slc2a4*, *Glut4*) was significantly down-regulated after GH-V treatment (0.92 ± 0.14 vs 0.45 ± 0.08 , $p < 0.05$). However, GH-V treatment did not alter the expression of hepatic insulin receptor substrates (*Irs*)-1, insulin receptor (*Insr*), v-akt murine thymoma viral oncogene homolog 3 (*Akt3*), *Igf-1*, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*Pik3ca*), phosphatidylinositol 3-kinase regulatory subunit alpha (*Pik3r1*).

Discussion

Recombinant GH-N therapy has long been used as an effective treatment for promoting growth due to its somatotrophic properties. However, treatment increases insulin resistance and alters carbohydrate and lipid metabolism (27). Placental variant GH-V is secreted from the placenta during human pregnancy and may also be associated with fetal growth in humans (8-12). Previous studies have observed growth-promoting properties of 22 kDa GH-

V in rodents (7, 13, 14, 28). However, these studies were conducted in non-pregnant animals. The aim of the current study was to evaluate the physiological effects of GH-V administration in a mouse model of pregnancy.

Despite previous reports of growth-promoting effects in non-pregnant mice, we did not observe any difference in maternal or fetal weight with increasing GH-V dose, although fetal crown-rump length was reduced in the 5 mg/kg treatment group. This is consistent with a study by Naar *et al.* who observed reduced fertility with compromised fetal growth in transgenic mice overexpressing human GH-V (29). Other studies have investigated the effect of maternal GH treatment on fetal growth during pregnancy with variable outcomes. Zamenhof *et al.* treated pregnant rats with bovine GH (3 mg/day) from day 7 to 20 of pregnancy, with no change in fetal weight but a significant increase in brain weight (30). Gargosky *et al.* treated pregnant rats with recombinant human GH (2.4 mg/kg per day) or human IGF-1 (1.4 mg/kg per day) via an osmotic pump but neither fetal or placental weight was affected by GH-N or IGF-1 treatment (31). In the sheep, Jenkinson *et al.* treated pregnant ewes with bovine GH-N during different stages of gestation and found that exogenous GH-N can stimulate fetal growth only after day 100 of gestation (32), while Harding *et al.* found that neither fetal or maternal growth was altered by bovine GH treatment from 125 days to 134 days of gestation (33). Discordant results on fetal growth have also been seen in pigs following GH interventions (34-37). It is likely that different GH preparations, dose regimens and treating periods may contribute to these findings. Moreover, nutrient partitioning may also play an important part in fetal growth (35, 38, 39) and it has been suggested that the anabolic effect of exogenous GH on the mother may counteract the growth-promoting effect of GH treatment on the fetus by reducing the nutrient supply (33, 40).

Previous studies have demonstrated that the placental lactogen is responsible for the maintenance of pregnancy and a series of actions include promotion of fetal growth in mice (41-43). As human growth hormone exhibits lactogenic activity in rodents (44, 45), it has been hypothesised that human GH administration may interfere with endogenous lactogen release in the rodent, or that high levels of GH act as an antagonist at the lactogen receptors but exhibit insufficient lactogenic effects during pregnancy (29, 46). Maternal glucocorticoid levels may also be involved in the effect of GH treatment on fetal growth. Increased maternal glucocorticoid levels impair fetal growth during pregnancy (47-50). As elevated glucocorticoid levels were observed in transgenic mice overexpressing the human GH gene (51, 52), maternal glucocorticoid may play a role in fetal growth following GH administration, although the chronic GH effects in transgenic mice may not be comparable to the relatively acute effects in GH administration during pregnancy. In fact, GH administration during pregnancy may elicit a number of interacting effects across the entire neuroendocrine systems. In our study, GH-V treatment did not promote maternal or fetal body weight but impaired fetal linear growth. Other possible mechanisms cannot be excluded.

Maternal adipose deposition and insulin levels

Surprisingly we observed a trend of increased maternal adipose tissue weight with increasing doses of GH-V. Although GH is widely recognised to have lipolytic properties, this has been debated. The controversy exists as it has been claimed that GH-N interacts with adipose tissue in different ways to promote both lipolytic and anti-lipolytic effects (28, 53, 54). In *in*

in vivo studies, it has been shown that GH-N administration reduces lipolysis and free fatty acids in both humans and animals (55-58), although this effect is transient and is only observed in the early period after GH-N injections with subsequent lipolytic effects (59-61). In addition, Kopchick *et al.* highlighted an increase in fat mass in young GH transgenic mice (≤ 6 months of age) followed by a reduction in adipose tissue in the older (62). GH-N and GH-V share similar structures and physiological effects. However, whether GH-V has similar actions on adipose tissue is largely unknown, especially during pregnancy, and the exact effects of GH-V on maternal adipose tissue remain unclear. In this study, we did not observe any significant differences in maternal adipose tissue weights after GH-V administration, although there were significant dose effects on adipose tissue weights.

Following GH-V treatment, we observed that maternal fasting insulin concentrations significantly increased at GD 18.5 with no corresponding changes in fasting glucose concentrations, suggesting that GH-V is a likely contributor to insulin resistance during pregnancy. Indeed, the GHR and insulin receptors share some signalling pathways, and both GH-N and GH-V stimulate phosphorylation of IRS following activation of Janus kinase-2 (63). Similar to the action of insulin, GH induces the tyrosyl phosphorylation of IRS proteins, providing binding sites for the regulatory subunits (p85) of phosphatidylinositol 3-kinase (PI3K) (64, 65). GH activation of PI3K via IRS phosphorylation plays an important role in glucose transport and lipid synthesis (66, 67). In transgenic mice, GH-V administration causes hyperinsulinemia by specifically increasing the protein expression of the p85 subunit in muscle and subsequently reducing PI3K signalling (68, 69). In our study, the mRNA expression of hepatic *Irs*, *Insr*, *Akt3*, *Pik3ca*, *Pik3r1* were unaltered after GH-V treatment. However, hepatic expression of the gene for insulin-sensitive glucose transporter 4 (*Slc2a4/Glut4*) was significantly down-regulated. Reduced expression of *Glut4* has been associated with insulin resistance and plays a role in the pathophysiology of type 2 diabetes mellitus (70). This may contribute to the insulin resistance induced by GH-V.

IGF-1 concentrations during pregnancy

IGF-1 is a primary mediator of the effects of GH, in particular its growth-promoting effects. Circulating IGF-1 is synthesized mainly by the liver under the control of GH. The binding of GH with its hepatic receptor stimulates expression and release of IGF-1 into the circulation (71). During human pregnancy, GH-V is secreted continuously from the placenta into maternal circulation from early pregnancy and rises exponentially until 37 weeks of gestation. Concomitantly, maternal GH-N falls to nearly undetectable levels. Maternal circulating IGF-1 concentrations increase dramatically in the second half of pregnancy with a peak at 37 weeks (5). It is believed that GH-V substitutes for GH-N to regulate maternal circulating IGF-1 concentrations during pregnancy (72, 73). However, conflicting results exist with regard to the relationship between maternal IGF-1 concentrations and fetal growth during pregnancy (74-76).

The effect of exogenous GH administration on IGF-1 levels has been investigated in previous animal studies. Treatment of rats with porcine or bovine GH increased maternal circulating IGF-1 levels (77, 78). Interestingly, rats treated with either recombinant human GH (31) or the 20 kDa isoform of GH-V (79) during pregnancy did not exhibit increased maternal IGF-1, although transgenic mice which overexpress human GH have increased IGF-1 during pregnancy or when non-pregnant (14, 29). Consistent with previous reports (30), we

observed that maternal IGF-1 increased during mid-pregnancy and decreased in late pregnancy. However, GH-V treatment did not affect maternal IGF-1 plasma concentrations at either GD 15.5 or 18.5. Some post-translational modifications, including specific cleavage, folding, subunit assembly, glycosylation, carboxylation *etc.*, may be responsible for this phenomenon (80).

In our study, we found that GH-V accentuated *Ghr* expression in the liver during mouse pregnancy; this did not affect the expression of *Igf-1*. This is consistent with studies by Jiang *et al.* who observed that bovine GH increased hepatic *Ghr* and *Igf-1* expression in cows (81), and Nilsson *et al.* who found that GH regulated *Ghr* mRNA levels in rat epiphyseal chondrocytes (89). Mathews *et al.* found no significant changes in hepatic *Ghr* mRNA levels between control and hypophysectomised rats treated with bovine GH, although pregnant females had elevated *Ghr* expressions (82). The time, dose and duration of GH exposure, *in vivo* or *in vitro* experiments, steroid hormones, and nutritional status may all contribute to the variations in results (83).

There are differences in the GH axis in mouse and human pregnancy which should be highlighted. As mentioned above, the human GH gene family is a cluster of five genes, which includes *GH-N*, *GH-V*, and the chorionic somatomammotropin (*CS-A*, *CS-B* and *CS-L*) genes (84). In contrast, the rodent genome contains a single pituitary-specific GH gene and lacks any GH-related CS genes (85). Consequently, only a pituitary version of GH is expressed during rodent pregnancy (86, 87). Circulating concentrations of pituitary GH increase during mouse pregnancy. Expression of the mouse GHR and GH-binding protein (GHBP) also dramatically increase; thus increased GHBP may decrease the availability of circulating GH. However, GHBP may also function as an important cell-surface receptor for GH in the liver (87). Furthermore, extra-pituitary expression of GH in multiple tissues is observed in both humans and mice, including the mouse placenta (88), suggests a potential role in mouse pregnancy. Although human GH-N and GH-V can both bind and activate the GHR of non-primate species, we cannot exclude species-specific differences.

In conclusion, GH-V administration did not affect maternal plasma IGF-1 concentrations or hepatic *Igf-1* mRNA expression but induced hyperinsulinemia in normal mouse pregnancy. Our results support the role of GH-V as a likely candidate to induce insulin resistance during pregnancy. Although GH-V treatment did not promote fetal growth in our studies, due to the intimate relationship between GH-V and fetal growth during human pregnancies, further investigation of specific animal models are warranted.

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