Mutations in the Glucokinase Gene of the Fetus Result in Reduced Placental Weight

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OBJECTIVE — In human pregnancy, placental weight is strongly associated with birth weight. It is uncertain whether there is regulation of the placenta by the fetus or vice versa. We aimed to test the hypothesis that placental growth is mediated, either directly or indirectly, by fetal insulin.

RESEARCH DESIGN AND METHODS — Birth weight and placental weight were measured in 43 offspring of 21 parents with mutations in the glucokinase (*GCK*) gene (25 had inherited the mutation and 18 had not), which results in reduced fetal insulin secretion. Birth weight, placental weight, umbilical cord insulin, and maternal glucose and insulin concentrations were measured in 573 nondiabetic, healthy, term pregnancies.

RESULTS — *GCK* mutation carriers were lighter and also had smaller placentas (610 vs. 720 g, $P = 0.042$). This difference was also seen in 17 discordant sibling pairs (600 vs. 720 g, $P = 0.003$). *GCK* mRNA was not detected in the placenta by RT-PCR. In the normal pregnancies, placental weight was strongly correlated with birth weight ($r = 0.61$, $P < 0.001$). Cord insulin concentrations were directly related to placental weight ($r = 0.28$) and birth weight ($r = 0.36$) ($P < 0.001$ for both).

CONCLUSIONS — These results suggest that insulin, directly or indirectly, plays a role in placental growth, especially as a mutation in the *GCK* gene, which is known to only alter fetal insulin secretion, results in altered placental weight. This finding is consistent with the preferential localization of the insulin receptors in the fetal endothelium of the placenta in the last trimester of pregnancy.

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The placenta plays a vital role in transporting nutrients from the mother to the fetus. Glucose, amino acids, and other substrates cross the placenta via a combination of active transport and facilitated diffusion, maintained by a plasma concentration gradient (1–3), which enables a continuous nutrient supply to the fetus. The nutrients have a direct effect on growth, and glucose has an indirect effect by stimulating fetal insulin, which is an important regulator of fetal growth (4,5).

In late gestation, placental and fetal size are closely correlated (6). Fetal insulin could represent a possible link between placental and fetal growth. Recently, significant correlations between measurement of umbilical cord insulin and placental weight have been observed (7,8). It is known that insulin is a major determinant of fetal growth, and it has been proposed that fetal insulin regulates placental growth as well (1). Insulin receptors are found on the maternal side of the placenta (syncytiotrophoblast) in the first trimester of pregnancy, but by term, the vast majority of insulin receptors are found on the fetal side of the placenta (in the endothelium), suggesting that they bind to fetal insulin rather than to maternal insulin in the last trimester of pregnancy (9,10). Insulin stimulates mitogenesis in placental cells (11); however, whether the insulin action cascade is initiated by the binding of maternal or of fetal insulin to the placental receptors is not known. Placental weights are increased in diabetic pregnancies, a condition known to be associated with fetal hyperinsulinemia (12–14), although this could be caused by the maternal hyperglycemic environment rather than fetal insulin levels.

Observations in monogenic disease in which a gene is mutated can give insights into normal physiology in humans. One example is mutations in the gene encoding glucokinase (*GCK*) that result in lifelong fasting hyperglycemia due to altered sensing of glucose by the pancreatic β-cell. These mutations, when present in a pregnant mother, result in an increase in offspring birth weight of 600 g, reflecting the impact of maternal hyperglycemia in pregnancy, which increases insulin-mediated growth in the fetus (15). When the same mutation is present in the fetus, it results in a reduction in offspring birth weight by 540 g, as the mutation reduces fetal insulin secretion and, hence, reduces insulin-mediated growth (15). These effects are additive, so a mother who has an offspring who inherits the same mutation will be of normal weight (15). As GCK specifically phosphorylates glucose to glucose 6-phosphate, the large weight changes in the fetus must reflect altered response to maternal glucose levels. Knockout animal studies have confirmed that the changes in birth weight associated with reduced GCK activity are mediated by fetal insulin (16).

We aimed to test the hypothesis that placental growth is mediated, either directly or indirectly, by fetal insulin by examining placental weight in offspring with and without *GCK* mutations. Offspring who inherit the mutation have reduced fetal insulin secretion as a result of their *GCK* mutation and therefore it would be possible to assess whether placental growth is lower in these pregnancies.

To ensure that any effect on placental weight reflects changes in fetal insulin re-

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Abbreviations: EFSOCH, Exeter Family Study of Childhood Health; GCK, glucokinase.

quires that GCK not be expressed in the placenta. Previous studies have not detected GCK in rodent placenta, but this result would not discount expression in the human. Therefore, we performed RT-PCR to rule out low-level expression in the human placenta.

Correlations of placental weight with cord insulin were also studied in a nondiabetic birth cohort. Subjects from this cohort provided a reference population for the GCK study.

RESEARCH DESIGN AND METHODS

Placental weight in *GCK* mutation pregnancies

Subjects were identified from the UK Maturity-Onset Diabetes of the Young referral database, stored at the Molecular Genetics Laboratory at the Royal Devon and Exeter Hospital (Exeter, U.K.), an international referral center for identifying *GCK* gene mutations. Women with pregnancies involving heterozygous mutations in the *GCK* gene were asked to provide details of each pregnancy resulting in a live birth, and permission was obtained to retrospectively review their obstetric case notes for each pregnancy.

Details of birth weight, gestational age at birth, and placental weight were obtained retrospectively from hospital obstetric records. Unfortunately, recording of the placental weight in hospital notes was not consistently performed in U.K. hospitals and measurements were only available for 43 (33%) births.

Genotype of the offspring was obtained using direct sequencing from a mouth swab if the child was aged <16 years or from a blood sample. Where possible, samples were taken by the investigators. If this was impractical, mouth swabs were performed by the parents of the children and sent back to Exeter via return mail; blood samples were taken by nurses at the patients' local health centers. Patients were not informed of genotyping results.

For this study, we present data on 43 live singleton births born to 21 parents with a mutation in the *GCK* gene (19 mothers). Of the offspring, 18 were unaffected (16 born to *GCK* mutation mothers) and 25 (23 born to *GCK* mutation mothers) were heterozygous for the familial *GCK* mutation. A subset of these data included 17 sibling pairs discordant for the *GCK* mutation.

Methods for *GCK* PCR

Biopsy samples from eight placentas were obtained at the time of elective cesarean section delivery (38.9 ± 0.4 weeks) of uncomplicated pregnancies. Full villous thickness specimens (~1 cm^3) were washed in sterile PBS, blotted to remove excess blood, and immediately snap-frozen in liquid nitrogen. For control specimens, a liver specimen obtained at the time of hepatic surgery for cirrhosis in adult men was obtained after pathological examination. Total RNA was prepared from whole tissue samples using a CsCl gradient. Placental samples were electrophoresed individually to verify RNA integrity, pooled, and reverse transcribed using a standard method (Stratagene, La Jolla, CA). Real-time PCR analysis was performed using a fluorescence temperature cycler (Lightcycler, Roche Molecular Diagnosis, Indianapolis, IN) with annealing temperature of 62°C for 30 cycles. PCR primers were forward 5′-CAT GAA GAG GCC AGT GTG AAG-3′ and reverse 5′-GAT GCC CTT GGT CCA GTT GAG-3′.

Normal reference population

Families were recruited as part of the Exeter Family Study of Childhood Health (EFSOCH) (17), a prospective study investigating genetic influences on fetal and early childhood growth. Parents were approached at the time of the antenatal booking visit and invited to take part if they were Caucasian and living in the EX1–EX4 postal code region of Exeter. Multiple pregnancies and women with diabetes were excluded. Informed consent was obtained from the parents of the newborns, and the study was approved by the North Devon Local Research Ethics Committee.

The full protocol is described elsewhere (17). In brief, mothers were seen by the research midwife at 28 weeks of gestation, and a fasting blood sample was taken for glucose and insulin measurement. Umbilical cord blood was obtained from the placental end of the cord after the placenta was delivered and centrifuged as soon as possible (18). Plasma aliquots were stored at −80°C. Cord plasma insulin and fasting insulin in both parents were measured using an immunochemiluminometric assay (Molecular Light Technology/Invitron, Monmouth, U.K.). The assay is specific for insulin, and the interassay coefficients of variation were <9.0% over the concentration range reported.

Babies were weighed (to the nearest 0.1 kg) within 24 h of delivery using Tanita electric scales. Placental weight (to nearest 25g) was measured using Brabantia scales. The placental ratio was calculated by placental weight (grams) divided by birth weight (grams) multiplied by 100 and expressed as a percentage. We present data on 573 families for whom placental weight and cord insulin results were available.

Statistics

Because of the smaller numbers in the *GCK* mutation group, nonparametric statistics were used, so data are presented as medians and interquartile ranges (IQRs), and differences between those with and without mutations in the *GCK* gene were assessed using the Mann-Whitney *U* test and Wilcoxon test (for sibling pair data). Associations in this group were assessed using Spearman's correlations. For the EFSOCH group, all data were tested for normality. Insulin results were log-transformed because of a skewed distribution. Data are presented as means ± SD or SEM for means adjusted for sex and gestation by ANOVA. Pearson correlation coefficients were used to assess associations in the EFSOCH data. Bonferroni adjustments to *P* values were made for multiple comparisons. Adjustments for gestational age and sex were performed using ANOVA and partial correlations. For consistency, comparisons between the *GCK* mutation and EFSOCH groups were performed using nonparametric statistics.

RESULTS

Placental weight in *GCK* mutation pregnancies

Table 1 shows the characteristics of 43 offspring born to 21 parents heterozygous for a mutation in the *GCK* gene, 25 of which had inherited the mutation (*GCK* NM) and 18 had not (*GCK* NN). The median gestational age was 38 weeks, predominantly because of medical intervention in birth for those babies classified as high risk because of maternal hyperglycemia/diabetes. Placental weights were similar for male and female babies (691 vs. 697 g, respectively, $P = 0.91$). Babies who did not inherit the mutation were significantly heavier at birth than offspring who did inherit the mutation (3.82 vs. 3.36 kg, $P = 0.007$). *GCK* NN babies had higher placental weights than *GCK* NM babies (720 vs. 610 g, $P = 0.042$). There was no difference in pla-

Table 1—*Characteristics of offspring of mothers with mutations in the GCK gene and characteristics of 118 babies in EFSOCH born at 38 weeks of gestation as a comparison group*

	GCK NN	*GCK* NM	*P* value	EFSOCH
n	18	25		118
Placental weight (g)	720 (650–834)	610 (540–788)	0.04	600 (500–700)
Placental ratio (%)	21.4 (17.0–22.9)	20.7 (18.7–24.4)	0.63	18.5 (16.4–20.6)
Birth weight (kg)	3.82 (3.25–4.32)	3.36 (2.59–3.71)	0.007	3.20 (2.94–3.53)
Male sex	10 (56)	13 (52)	0.82	65 (55)
Gestation (weeks)	38.0 (37.0–40.0)	38.0 (38.0–40.5)	0.36	38

Data are presented as median (interquartile range) or *n* (%). *P* values refer to the differences between *GCK* NN and *GCK* NM groups.

cental ratio between the two groups ($P = 0.63$). Insulin treatment was given in 11 of 23 diabetic pregnancies, resulting in affected babies, and in 6 of 16 diabetic pregnancies, resulting in nonaffected babies. There was no difference in placental weight between those treated with insulin and those not treated (median 680 vs. 680 g, respectively, $P = 0.69$).

Further analysis was performed on a subgroup of these babies consisting of 17 discordant sibling pairs from 11 nuclear families. Babies who had inherited the mutation had significantly lower placental weights than their unaffected siblings in 15 of 17 cases ($P = 0.02$ by χ^2) (median 600 g [IQR 475–652 g] vs. 720 g [650–810 g], respectively, $P = 0.003$) (Fig. 1) and lower placental ratios (18.9% [16.3–20.3%] vs. 21.6% [17.4–23.4%], respectively), although this difference did not quite reach significance ($P = 0.051$).

Expression of GCK in placenta

mRNA for GCK was not detected in placental villous tissue obtained in term pregnancies but was easily detected in the positive control (liver).

Normal birth cohort

In 573 normal pregnancies from the EFSOCH study, placental weight was highly correlated with birth weight ($r = 0.61$, $P < 0.001$) and cord insulin concentration ($r = 0.28$, $P < 0.001$). There was also a weaker but significant correlation between placental weight and maternal insulin ($r = 0.13$, $P = 0.021$). As expected, maternal glucose and maternal insulin were significantly correlated with cord insulin ($r = 0.26$ and $r = 0.20$, respectively, $P < 0.001$), and maternal glucose and cord insulin were significantly associated with birth weight ($r = 0.27$ and $r = 0.36$, respectively, $P < 0.001$).

We examined a subgroup of 118 births with gestational age of 38 weeks to serve as a reference population for the *GCK* mutation group (Table 1). Median (IQR) placental weight was 600 g (500–700 g), which was lower than the placental weights of the babies that had not inherited the *GCK* mutation ($P < 0.001$), which is what may be expected with the large proportion of hyperglycemic/diabetic pregnancies. However, this result was similar to the placental weights of babies who had inherited the mutation ($P = 0.26$) (Fig. 2).

CONCLUSIONS — Our study of placental weight in the offspring with and without heterozygous mutations in the *GCK* gene supports the hypothesis that fetal insulin may directly or indirectly regulate placental growth. We found significant correlations of placental weight with umbilical cord insulin concentrations and birth weight, confirming relationships seen in previous studies (7,8) and in agreement with data from diabetic pregnancies (12–14). The GCK study gives us more insight into the direction of the causal relationship. In the *GCK* mutation pregnancies, the weight of the placenta, like birth weight, depended on the mutation status of the fetus. We have shown that the babies who inherited the mutation had a lighter placenta than the babies without the mutation. This finding was demonstrated most clearly in the discordant sibling analysis (Fig. 1), but a signif-

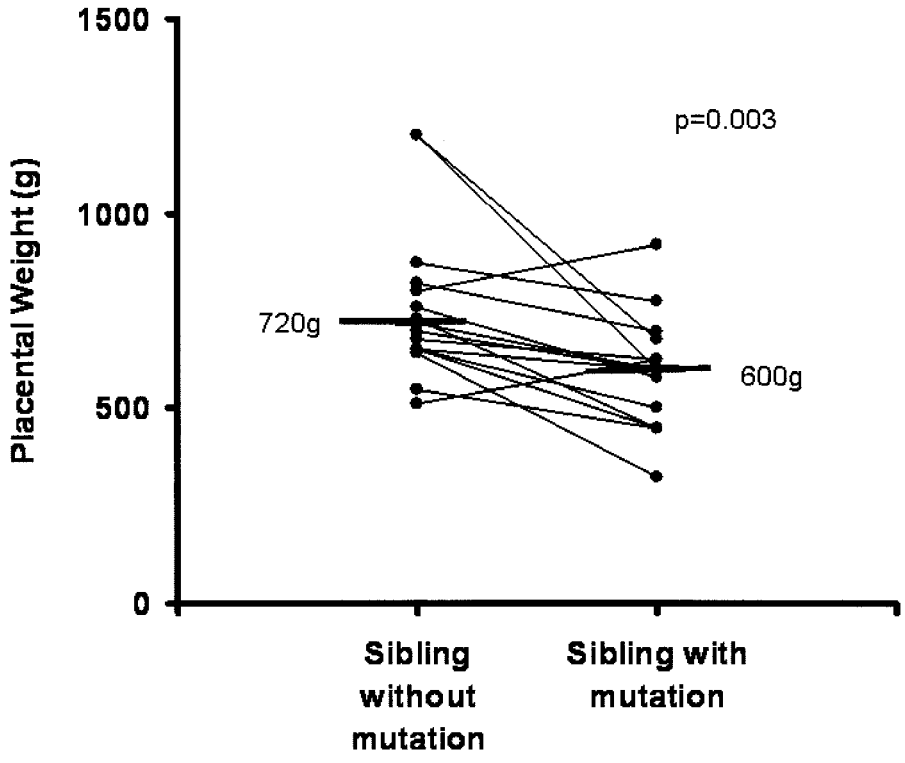

Figure 1— *Placental weights of 17 sibling pairs discordant for a mutation in the GCK gene. The median of each group is represented by a horizontal line. Birth weight was lower in the sibling with the mutation in 15 of 17 sibling pairs (P = 0.02).*

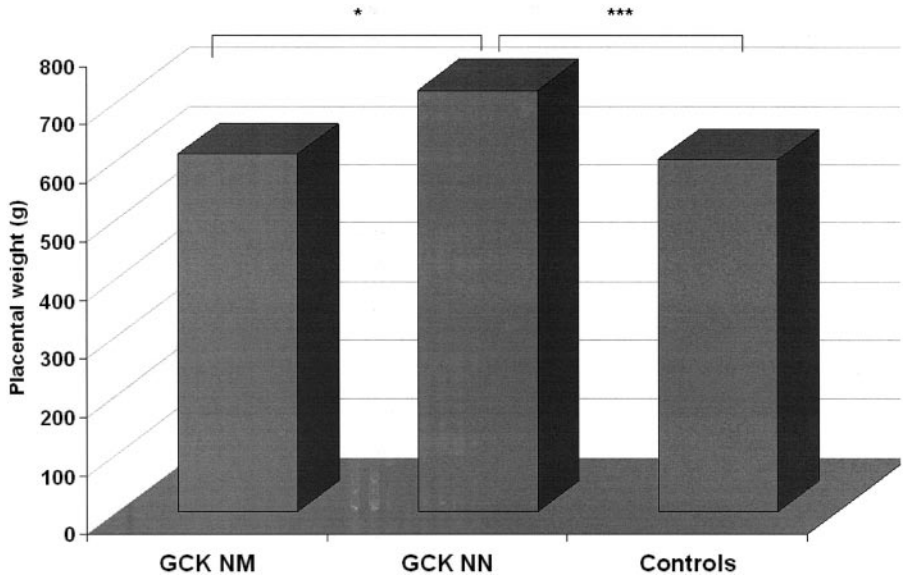

Figure 2— *Bar chart showing median placental weight for* GCK *NM babies,* GCK *NN babies, and control babies born at 38 weeks of gestation from the EFSOCH study.* ***P $<$ *0.001;* *P $<$ *0.05*

icant difference was also seen when all babies were compared with all controls, showing that the presence of the mutation results in a smaller placenta. This result is similar to the previously observed effect of inheriting a *GCK* mutation on offspring birth weight (19).

GCK is the pancreatic glucose sensor, and mutations in the gene have been shown to cause β-cell dysfunction resulting from altered glucose sensing with a reduction of the insulin secretion rate for a given glucose value (19). Although cord insulin values were not measured in the *GCK* mutation births, there are animal data suggesting that the fetus with a heterozygous knockout and reduced GCK activity has reduced insulin secretion and hence reduced insulin-mediated growth effects (16).

We propose that the differences in placental size between *GCK* NM and *GCK* NN babies were mediated either directly or indirectly through reduced fetal insulin secretion. GCK was shown not to be expressed in the rat placenta using Northern blot analysis (23), and we showed using a highly sensitive RT-PCR method that no mRNA from the *GCK* gene can be detected in term placenta. The absence of placental GCK expression was further supported by the absence of signal in microarray analysis of 15 term placentas (data not shown). This means that the impact of the presence of a *GCK* mutation must be mediated through the change in fetal GCK activity, possibly through changes to fetal insulin secretion having a direct impact on placental growth. In keeping with this hypothesis, in the third trimester of pregnancy, the insulin receptors are more abundant in the vascular endothelial cells facing the fetal systemic circulation than in those on the maternal side (12). Alternatively, decreased fetal insulin concentrations could have an indirect effect on placental mitogenesis with reduced secretion of insulin reducing fetal growth and a factor or a cascade of signals other than those elicited by fetal insulin resulting in the reduction in placental size.

There were a number of limitations in these studies. First, maturity-onset diabetes of the young is relatively rare; therefore, we only had small numbers in the *GCK* mutation group. However, we have replicated results from previous small studies that have shown relationships between birth weight and *GCK* mutations (15,20), and the results from both studies presented here are internally consistent. As insulin was not measured in the patients with *GCK* mutations, we have had to make assumptions about fetal insulin on the basis of surrogate markers of fetal birth weight and animal studies. We cannot exclude a coincidental problem in the placental vasculature, but this is very unlikely, especially because fetal GCK is not detected in the placenta and the placentas of the fetuses with and without the mutation were exposed to a similar degree of maternal glycemia, and data were consistent with the insulin receptors being present predominantly in the fetal endothelium in the last trimester of pregnancy.

In summary, we have data from normal and monogenic pregnancies that support the hypothesis that fetal insulin can regulate placental weight.

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