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Placental insulin-like growth factor II (IGF-II) and its relation to litter size in the common marmoset monkey (*Callithrix jacchus*)

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Abstract

The primate placenta produces a wide variety of hormones throughout gestation that regulate placental function and fetal growth. One such hormone is insulin-like growth factor-II (IGF-II), a peptide implicated in cell division, differentiation, and amino acid transport. IGF-II concentrations were measured in 23 common marmoset (*Callithrix jacchus*) term placentas from twin and triplet litters in order to determine whether previously described differences in fetoplacental phenotype such as placental and litter mass and placental surface area were related to differences in endocrine function. IGF-II was extracted from frozen tissue samples and measured using an enzyme-linked immunosorbent assay kit designed for human tissue which was validated for marmoset placenta. IGF-II concentrations were not related to placental or litter mass, and twin and triplet placentas did not differ in total concentration. However, per individual fetus, triplets were associated with a significant 42% reduction in IGF-II concentration ($p=0.03$), and IGF-II concentration per gram of fetal mass was a third lower in triplet litters. The triplet placenta exhibits a global expansion of the surface area which was contrasted by a per unit area reduction in IGF-II concentration ($r=-0.75$, $p=0.01$), a pattern which explains why twin and triplet placentas overall did not differ in concentration. Per fetus, triplet pregnancies are associated with relatively less maternal mass, placental mass, microscopic surface area, suggesting that the intrauterine growth of triplets is supported by systems that increase the efficiency of nutrient transfer. The finding that individual triplet fetuses are also associated with significantly lower IGF-II concentrations is consistent with the view that the marmoset fetoplacental unit exhibits a flexible pattern of placental allocation and metabolism. Plasticity in placental endocrine and metabolic function is likely to play an important role in the ability of the fetus to sense and accommodate the intrauterine environment, and by extension, the external ecology.

Keywords

placenta; litter size; *Callitrichidae*; endocrinology; plasticity

INTRODUCTION

Placental and fetal growth and development are complicated, interconnected phenomena, the coordination of which is regulated by an equally complicated network of hormonal factors [Murphy et al. 2006]. The insulin-like growth factors-I and -II are both 7.5 kDa polypeptide hormones that are found in a wide range of fetal tissues, including the placenta, and act as growth promoters in a variety of placental cell types [Jones and Clemmons 1995]. IGF-II is specifically involved in the proliferation, invasion, and differentiation of the placenta, particularly of the syncytiotrophoblast, the placental cell layer in direct contact with maternal circulation [Chakraborty et al. 2002; Han and Carter 2000; McKinnon et al. 2001]. Circulating IGF-II is associated with both birth weight and placenta weights, and low concentrations of IGF-II are related to intrauterine growth restriction (IUGR) in humans and animal models [Fowden 2003; Gicquel and LeBeuc 2006]. However, few studies have examined relations between birth weight, placental weight, and IGF-II in non-human primates.

Common marmosets produce dizygotic litters ranging from singletons to quadruplets in captivity [Tardif et al. 2003]. All marmoset littermates, regardless of litter size, share two placental discs integrated by a vascular network that leads to shared placental circulation [Benirschke et al. 1962; Wislocki 1932; Wislocki 1939] and thus, shared access to maternal resources [Rutherford and Tardif 2008]. Triplet marmoset pregnancies can be considered a nutrient-restricted state [Rutherford and Tardif 2008], paradoxically in the context of maternal abundance. Ovulation number and litter size at birth are positively associated with maternal weight [Tardif and Jaquish 1994], but triplets are born at significantly lower birth weights [Chambers and Hearn 1985; Rutherford and Tardif 2008]. Despite the association between higher maternal mass and triplet litters, individual marmoset fetuses have a reduced share of that maternal mass, as well as less placental mass, compared to twins [Rutherford and Tardif 2009]. The association between per capita reductions in access to maternal resources and lower birth weights supports the argument that the triplet marmoset fetus experiences a nutrient-restricted intrauterine environment. Further, individual triplet marmoset fetuses are associated with less microscopic surface area of the placenta [Rutherford and Tardif 2009], which is the primary interface for nutrient transport. This surface area is also the site of IGF-II production, indicating that variation in litter size in the common marmoset may be associated with variation in placental IGF-II.

The aims of this study were to determine a) whether an assay for human urinary and serum IGF-II could be validated for use in the marmoset placenta, and if so, b) whether concentration of IGF-II varied with respect to litter size or weight or placental weight. The null hypothesis tested is that IGF-II concentrations will not differ between full-term placentas from different litter categories, but rather will reflect a “steady-state” for the marmoset placenta. The alternate hypothesis is that IGF-II concentration will vary in ways that differentiate the quality of the intrauterine environment between the twin and triplet marmoset pregnancies.

METHODS

Placental samples

This study was conducted within the bounds of Institutional Animal Care and Use Committee- approved protocols at the Southwest National Primate Research Center in San Antonio, Texas. Twenty-three term placentas from 12 triplet litters and 11 twin litters were assayed for tissue concentration of IGF-II. Placental tissue used in this study was collected from the common marmoset colony at the Southwest National Primate Research Center from 1997 to 2006. Placental collection was opportunistic and occurred at night at the time

of natural parturition (usually between 20:00 h and 01:00 h) [Rutherford and Tardif 2008], from pregnancies of normal gestation length (148 ± 4 days for *Callithrix* [Hearn and Lunn 1975]). Only intact (i.e. uneaten) placentas from liveborn litters were included in this study. The placentas were collected as soon after delivery as was possible (usually within 10 minutes) and weighed within an hour of collection. Since the marmoset placenta is bidiscoid [Wislocki 1932], one disk was randomly selected for formalin fixation for histological evaluation [Rutherford and Tardif 2009] and the other was placed in a plastic WhirlPac™ sample bag and stored in a -4°C laboratory freezer immediately after weighing. Neonates were weighed within 24 hours of birth. Mothers were weighed regularly as part of normal colony protocol; non-pregnant weight refers to weight taken either immediately before or 1–2 months after the pregnancy being studied.

IGF-II Extraction

Frozen samples were shipped on dry ice to the Center for the Integrative Study of Animal Behavior Laboratory at Indiana University where they were stored at -4°C and subsequently extracted. We followed a protocol developed by [Bowman et al. 2001] and modified for use with commercial extraction reagents provided in the Diagnostic Systems Laboratories, Inc. (Webster, TX) IGF-II extraction kit (DSL-10-9100). Approximately 0.4 g pieces of tissue were cut from the placenta, placed in foil, flash frozen with liquid N_2 , and then pulverized with a ceramic pestle. The pulverized tissue was placed in 1 mL Eppendorf vials. 750 μL of an acid-ethanol extraction solution (12.5% 2N HCl/87.5% absolute ethanol) provided in the kit was added to the tissue; the tube was then vortexed and incubated at room temperature for 30 minutes. After incubation, tubes were centrifuged at room temperature for 3 minutes at 10,000 rpm. 100 μL of the supernatant was combined with 500 μL of a neutralizing buffer solution provided in the kit, and vortexed.

IGF-II Assay

Samples were assayed using the DSL-10-9100 ACTIVE® IGF-II ELISA kit (Diagnostic System Laboratories, Inc., Webster, TX) for human serum. The assay cleaves IGF-II from its binding proteins, thus yielding a concentration of total IGF-II. This “two-step” sandwich-type immunoassay involved two antibody steps: first, 20 μL of controls, standards, and samples of unknown concentration were incubated with 100 μL of assay buffer in microtitration wells coated with an anti-IGF-II antibody; second, after incubation and washing, 100 μL of an anti-IGF-II detection antibody labeled with the enzyme horseradish peroxidase (HRP) was added to the wells. After incubation and washing, we added 100 μL of a chromagen solution (tetramethylbenzidine) to develop the color in the wells. After this final incubation, an acid-based color stopping solution was added, and the plate was analyzed by dual wavelength absorbance to determine the rate of enzymatic turnover. Absorbance is directly proportional to IGF-II concentration. A curve was plotted between the absorbance values and IGF-II concentration of the known standards; samples with concentrations that fell beneath the minimum value of the standard curve were discarded from further analysis. All assays were run in duplicate. We also included duplicate assay blanks, which were subtracted from each sample prior to analysis.

Kit validation for marmoset tissue

The kit used was developed for human serum, and so we attempted to validate it for marmoset tissue samples. This kit protocol included a seven-point standard curve, as well as duplicate blanks, standards, and non-specific binding controls. Cross-reactivity of the kit with other related ligands such as IGF-1, insulin, and IGF binding proteins 1 and 3 was non-detectable. The sensitivity of the assay was 0.25 ng/ml IGF-II.

Extraction efficiencies of known concentration samples were measured as the percent IGF-II recovered in extracted compared to non-extracted controls. Efficiencies averaged 22.81%. Serial dilutions ranging from 1:2 to 1:10 were performed for a subset of marmoset placental samples and confirmed both linearity and parallelism with the standard curve ($p>0.9$, Figure 1). The interassay coefficient of variation for a known IGF-II control (286 ng/ml) was 10.9%, and the intraassay coefficient of variation was 3.4%. The intraassay coefficient of variation for tissue extracts was 2.81%.

Microscopic morphometry of the marmoset placenta

Rutherford and Tardif (2009) measured the microscopic surface area of the marmoset placenta using stereological methods. Stereology employs a system of test grids that are projected on two-dimensional histological slides, and that are used to count points overlying tissue compartments of interest in relation to the total number of points intersecting with the entire tissue section [Mayhew 2006]. This generates simple percentages of tissue types that can be converted to surface area and volumetric units through multiplication by the total tissue volume measured at the time of collection. For the analyses here, we assessed IGF-II concentration in relation to the amount of surface area of the placenta that interfaces with maternal circulation, as this is the placental component responsible for IGF-II production.

Statistical analyses

Repeatability of both ovulation number ($r=0.08$) [Tardif and Jaquish 1997] and litter size ($r=0.128$) [Jaquish et al. 1991] within individual females is low for marmosets, suggesting low heritability for litter size. Further, maternal mass is not related to placental mass [Rutherford and Tardif in press]. For these reasons, all placentas in these analyses are treated as independent events. All data were confirmed for normality before parametric tests were applied. Pearson's correlations were performed to analyze relations between IGF-II concentrations and placental, neonatal, and total litter weights, as well as microscopic morphological variables for a subset of 10 placentas. To assess whether IGF-II concentration in relation to individual fetuses differed, concentration was divided by 1) litter number and 2) litter weight to generate two variables. A median split was applied to both placental weight and total litter weight to characterize differences in IGF-II concentration between 1) light and heavy litters and 2) light and heavy placentas. T-tests were used to assess differences in IGF-II characteristics between litter and placental categories.

RESULTS

Concentrations are reported as mean ng IGF-II/mL placental extraction (S.D.). The mean IGF-II concentration in the marmoset placenta is 579.86 (341.64) ng/mL. Concentration exhibited no significant correlations with placental weight, total litter weight, or average neonate weight (Table 1). Twin and triplet placentas did not differ significantly in IGF-II concentration (Figure 2a; $t=0.61$, $p=0.55$). IGF-II concentration did not differ between lighter and heavier small placentas (Figure 2b, $t=0.85$, $p=0.41$), nor between lighter and heavier litters (Figure 2c, $t=0.29$, $p=0.77$).

Whereas twin and triplet placentas did not differ in terms of total IGF-II concentration, the relationship between individual fetuses and IGF-II concentration varied significantly according to litter size (Figure 3a). Individual triplets were associated with a significant reduction in IGF-II concentration compared to their twin counterparts ($t=2.33$, $p=0.03$). Per gram of litter mass, triplet placentas tended to produce lower concentrations of IGF-II compared to twin placentas (Figure 3b; $t=1.49$, $p=0.15$).

Data for microscopic placental surface area and IGF-II concentration were available for 10 placentas (twin, $n=6$; triplet, $n=4$). Overall, there was a highly significant negative relation between IGF-II concentration and microscopic surface area (Figure 4, $r=-0.753$, $p=0.01$).

DISCUSSION

The present study is the first known to confirm the presence of IGF-II in the marmoset placenta and thus the first to directly consider the relation of IGF-II to marmoset fetal and placental growth. The first important finding of the study is that IGF-II can be detected in marmoset placental tissue using simple extraction methods and an assay designed for detection of human IGF-II and validated for use in marmoset tissue. This alone provides a critical foundation for future studies of marmoset pregnancy regarding specific localization of IGF-II production, total expression and production levels, and interactions with IGF-II binding proteins and receptors, as well as other hormone systems.

The second set of results characterizes patterns of variation of IGF-II concentration. On the gross level (i.e. whole litter, whole placenta), marmoset placental IGF-II concentration does exhibit a “steady state”. There were no significant differences in IGF-II concentration according to litter size, litter mass, or placental mass. However, it is intriguing that although not significant, the association of IGF-II concentration with litter size and mass, and placental mass was negative. Triplet litters, heavier litters, and heavier placentas all tended to have lower IGF-II concentration. This pattern is fully realized at the level of the individual fetus. Individual triplets (at term) are exposed to 42% lower placental IGF-II concentrations than are individual twins, with each gram of fetal mass supported by an IGF-II reduction of nearly a third. Therefore, the lack of difference in concentration at the level of the litter or the whole placenta obscures crucial differences in placental function related to fetal growth.

This clear difference at the fetal level between twin and triplet production of and/or exposure to placental IGF-II is mirrored by other important functional litter-based differences in the marmoset fetoplacental unit. The triplet marmoset placenta is not significantly heavier than the twin placenta, despite nearly a 30% increase in total neonatal mass in triplet litters [Rutherford and Tardif 2008]. Thus, although all littermates regardless of litter size share an integrated placental mass, the individual share of the placenta for a triplet fetus is significantly smaller than that of its twin counterpart. The result of this is a significantly higher fetal:placental weight ratio in triplet litters compared to twins such that one gram of triplet placenta is associated with 25% more fetal mass than one gram of twin placenta [Rutherford and Tardif 2008]. The present findings are consistent with this pattern of differential placental allocation and function. It is important to note that while all littermates share the placenta, it is as yet unknown how exactly this mass is allocated to individual fetuses via the circulatory connections or perhaps endocrine signaling pathways. It may well be that there are distinct divisions of placental regions and attendant function to individual littermates that add to the complexity of these analyses.

We carried the analysis to the microscopic level and demonstrated a highly significant inverse relationship between surface area and IGF-II concentration. The surface area reflects the amount of placental tissue, the syncytiotrophoblast, that is available for IGF-II synthesis; placentas with large surface areas produce lower amounts of IGF-II. The four placentas that had both the lowest IGF-concentrations and the greatest surface area were all from triplet litters. Although the triplet placenta as a whole exhibits a 40% expansion of this surface area compared to twin placentas (controlled for overall placental volume), individual triplet fetuses experience a significantly reduced share of the this interface, which is the site of IGF-II production [Rutherford and Tardif 2009]. This is consistent with a per-fetus reduction

in IGF-II concentration, and may explain why overall concentration does not differ between twin and triplet placentas broadly.

That there is an overall expansion of the surface area but reduced IGF-II concentration per unit area of the triplet marmoset placenta suggests an important alteration in metabolic function underlying fetal growth and possibly intrauterine growth retardation in triplet fetuses. In mice, deletion of the placental-specific promoter of the *igf2* gene yields smaller placentas, but significantly higher fetal:placental weight ratios [Constancia et al. 2002]. These low-IGF-II, low-weight placentas had reduced passive amino acid transport, but active transport by the system A transport system was increased, and as a result, the placenta increased its efficiency in the production of fetal mass, suggesting that in the face of impaired IGF-II production the placenta may initiate other acquisitive mechanisms designed to compensate for this deficiency. However, increased efficiency fails at some point in the mouse model, and neonates born to the *igf2* PO knockout placentas are smaller than normal [Constancia et al. 2002]. In marmosets, fetal weights are similar in twins and triplets until the final weeks of gestation [Chambers and Hearn 1985]. This may reflect comparable IGF-II regulation of fetoplacental growth early in gestation, before nutritive or oxygen requirements or other stressors constrain triplet growth. However, as fetal growth and the attendant metabolic demands increase, IGF-II production may drop. In the triplet marmoset placenta, there may be a reorganization of transport capacity such that passive transport decreases while active transport increases even when IGF-II is reduced.

We are limited by these data in our ability to characterize the functional range of the full placental IGF system during pregnancy. First, concentration data must be interpreted cautiously. Tissue hormone concentration is not analogous to circulating concentration levels in plasma, nor is it accurate to equate increases in protein concentration with increases in protein production or increases in gene expression. Second, IGF-II acts in concert with IGF-I, IGF receptors and binding proteins, and other molecular and metabolic drivers of fetoplacental growth which may refine the explanation of the lack of an association between placental mass and IGF-II concentration as well as the inverse relation between surface area and IGF-II. A more complete analysis of all components of this dynamic system, collected from placental tissue as well as maternal and/or fetal/neonatal urine and serum, is clearly necessary.

Since the tissue assayed was from term gestations only, it is difficult to speculate on the ontogeny of IGF-II production and its impact on longitudinal placental and fetal growth in the marmoset. It is possible that fetal development is mapped onto the timing of placental IGF-II production in ways that differentiate twin and triplet intrauterine growth trajectories. Microscopic analysis of marmoset placental morphology at term indicates an important structural divergence between twin and triplet placentas during the last trimester, a time at which the growth-restricted phenotype of the triplet fetus emerges [Rutherford and Tardif 2009]. These growth dynamics are likely related to IGF-II profiles that may not be reflected by concentrations in term placental tissue. If there are mechanisms that dampen IGF-II production near term to limit fetal size in triplet pregnancies, then IGF-II at term will be an incomplete reflection of the developmental dynamics of placental growth throughout gestation.

Whereas total IGF-II concentration is apparently not related to broad aspects of the marmoset fetoplacental unit such as litter size and weight and placental mass, there is a clear differentiation at the level of the individual fetus between twin and triplet litters, and at the level of the microscopic functional placental architecture that suggest divergence and plasticity in nutrient allocation pathways and growth trajectories. Plasticity in placental endocrine and metabolic function is likely to play an enormously important and potentially

central role in the ability of the fetus to flexibly sense and accommodate the intrauterine environment, and ultimately the external ecology [Rutherford 2009]. Elucidating the determinants and consequences of IGF-II concentration and the direct effects of IGF-II on trophoblast growth and differentiation in the marmoset placenta and on the long-term sequelae of the intrauterine environment are targets for future study.

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Figure 1.
IGF-II assay validation for marmoset tissue

Figure 2.

There were no significant differences in IGF-II concentration between (a) twin and triplet placentas, (b) lighter and heavier placentas*, or (c) lighter and heavier litters**. (*Median split at 8.38 g; ** Median split at 69.56 g)

Figure 3a: IGF-II concentration per individual fetus

Figure 3b: IGF-II concentration per gram neonatal mass

Figure 3.

Figure 3a: Individual triplet fetuses were associated with significantly lower IGF-II concentrations than twins.

Figure 3b: Triplet placentas produce lower IGF-II concentrations per gram of litter mass (n.s.)

Figure 4. IGF-II concentration and microscopic placental surface area are significantly inversely related.

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Table 1
Correlations between IGF-II concentration and components of the fetoplacental unit in the marmoset

Placental mass		Total litter mass	Average neonate mass	Maternalnon-pregnant mass
IGF-II concentration	r	-0.05	0.008	0.274
	p	0.81	0.97	0.44