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The role of insulin, insulin-like growth factors I and II, insulin-like growth factor binding protein 3, and their receptors in the regulation of human fetal growth

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A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Dai-Trang Elizabeth Le

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DEDICATION

To my family for their love and continuous support and to my friend, Sean O'Neill, for his words of encouragement and belief in me.

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ABSTRACT

THE ROLE OF INSULIN, INSULIN-LIKE GROWTH FACTORS I AND II, INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 3, AND THEIR RECEPTORS IN THE REGULATION OF HUMAN FETAL GROWTH. D. Elizabeth Le (Sponsored by Harold R. Behrman). Department of Obstetrics and Gynecology, Yale University, School of Medicine, New Haven, CT.

This investigation was undertaken to determine the relationship between normal human fetal growth and the levels of insulin, insulin-like growth factors I and II (IGF-I and IGF-II), insulin-like growth factor binding protein 3 (IGFBP-3), and their receptors in both the maternal and fetal compartments.

Serum (n=20) and amniotic fluid (AF) (n=7) samples were obtained from normal pregnant women, their term neonates (n=20) between 38–41 weeks gestation, and fetuses (n=32) via cordocentesis between 21–36 weeks gestation. Neonates were designated as appropriate for gestational age (AGA) if their weights were between the 10th and 90th percentiles and large for gestational age (LGA) if they were greater than the 90th percentile. Newborns were also classified as small or large if their weights were below or above the 50th percentile for newborns of the same age. Serum and AF samples were analyzed for levels of insulin, IGF-I, IGF-II, and IGFBP-3 by radioimmunoassay. Insulin and type 1 IGF receptors were assessed by immunohistochemistry from placental tissues.

Fetal serum IGF-I, IGF-II, and IGFBP-3 remained stable between 21–36 weeks. At term, large neonates had increased IGF-I and IGFBP-3 levels compared to levels measured during 21–27 weeks and 28–36 weeks gestation ($x \pm SEM$; [IGF-I] 53 $\pm$ 8

ng/ml and 58 ± 7 ng/ml vs. 86 ± 6 ng/ml, $p<0.05$; [IGFBP-3] 0.08 ± 0.08 μg/ml and 0.08 ± 0.05 μg/ml vs. 1.10 ± 0.04 μg/ml). There was a direct correlation between gestational age and birth weight and the levels of IGF-I ($r=+0.297$, $p<0.05$; $r=+0.564$, $p<0.02$, respectively) and IGFBP-3 ($r=+0.386$, $p<0.01$; $r=+0.503$, $p<0.002$, respectively). IGF-I, IGF-II, and IGFBP-3 levels, but not insulin, were significantly higher in large neonates compared to small neonates. Insulin and type 1 IGF receptors were detectable in placental membranes as early as 7 weeks gestation.

These data demonstrate the bioavailability of fetal IGF-I, IGF-II, IGFBP-3, and type 1 IGF receptors throughout pregnancy. Fetal serum IGF-I and IGFBP-3 were shown to be associated with fetal age and birth weight in normal pregnancies.

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INTRODUCTION

Normal Fetal Growth

Human fetal growth consists of forty-four cell divisions from the time the ovum is fertilized to delivery at term and is characterized by three phases: hyperplasia, hyperplasia with concomitant hypertrophy, and hypertrophy. [9,64] By the third week, the embryo is composed of three germ cell layers and from the fourth to the eighth week, organogenesis takes place. By the end of the 16th week, the intestines have returned to the abdominal cavity and the genitalia are well-defined. During this period, all of the limbs have developed with ossification also in progress.

During the preconceptional period, genetics, maternal nutritional state, and biological and environmental factors may affect subsequent development of the conceptus. The mechanism of genetic control of cell growth and differentiation that is the determinant of species size at birth is poorly defined. Approximately 20% of birth weight is attributable to fetal genotype. The male sex is associated with increased birth weight of 150–200 gm when compared to the female sex. Another 20% of the variation is due to maternal influence including race, age, height, weight, and parity. [64] During the embryonic period (conception through the first 8 weeks) teratogens and uncontrolled maternal diabetes can change normal growth patterns as this is the period of organogenesis. From the end of the embryonic period through delivery, nutrition, [95] hypoxia, [57] and placental, hormonal, environmental, [109] and genetic factors can alter growth.

The birth weights of infants delivered at various gestational age follow a growth curve in which fetal weight increases exponentially until the latter part of gestation, when fetal growth rate slows leading to a sigmoidal-shaped curve. The curve shows a steady rate of increase from approximately 24 weeks to 37–39 weeks, and in most series, there is flattening of the curve at 37–39 weeks. [60, 103] In general, the rate of growth is slower after 30 weeks of gestation in twins and after 36 weeks in singletons. After 44 weeks, there is a decrease of average birth weight. [91]

Acceleration of fetal weight during the last trimester is manifested by an 11-fold increase in fat, 3-fold increase in protein, and an overall 2.5-fold in weight. [91] The fetus grows at approximately 1.5% per day or 15 gm/kg/day during this period. Nonfat dry weight increases at approximately 2.25 gm/kg/day and fat accretion increases at approximately 3.5 gm/kg/day, although the latter is more variable. The remainder of the 15 gm represents water accretion. [64]

The fetal liver is the major site for protein synthesis in the latter part of gestation and is regulated by substrate availability and modulation of synthetic apparatus by endocrine and other factors. Glucose is the major substrate utilized by the growing fetus and is stored in the fetal liver as glycogen; it is transplacentally distributed in the fetal circulation via facilitated transport. Glucose storage dramatically increases after 36 weeks for enhanced glycolysis capacity for the fetus during periods of stress in the intrapartum and early newborn periods. [64]

Placental growth, which follows the same three phases of development as the fetus, is also critical for fetal growth. Normal placentas increase in size almost linearly

up to 36 weeks and are influenced by human placental lactogen (hPL) and human chorionic gonadotropin. Maternal serum hPL increases with gestational age until it plateaus at 37 weeks with a slight decline between 37–40 weeks. One of its action is to block maternal utilization of glucose and to promote mobilization and utilization of free fatty acids thereby increasing the amount of glucose available to the placenta for transfer to the fetus. [22] Uteroplacental constraints appear to become the major factor for growth at 3000–3200 gm in normal pregnancy. [74]

Deviant Growth

Neonates are designated as small for gestational age (SGA) when they are less than the 10th percentile for birth weight, appropriate for gestational age (AGA) when they are between the 10th and 90th percentiles, and large for gestational age (LGA) when they are greater than the 90th percentile. However, statistically, 10% of infants should be below the 10th percentile and 10% should be above the 90th percentile regardless of medical interventions. This group, therefore, may reflect biological diversity as well as deviant growth.

Intrauterine growth retardation (IUGR) is defined as transient or persistent decrease in the rate of fetal growth that exceeds a given percentage of either the earlier individual growth rate or the normal expected growth rate. Type I growth restriction results in symmetric reduction in growth of all tissues because malnutrition occurs during hyperplasia leading to organs that are permanently reduced in cell number. The fetus therefore is not necessarily underweight for height. In Type II growth restriction,

undernutrition occurs during the hypertrophic phase leading to reduction in cell size. It does not become significant until the third trimester. Normal fetal length is usually preserved with head sparing growth and soft tissue wasting. This process is reversible with improved nutrition. [15]

Early menarche, low prepregnancy weight and height, and short interpregnancy intervals have been associated with increased risk of delivering small babies. Delivery of growth retarded infants predisposes to growth retardation in subsequent pregnancies. [31]

From 36 weeks gestation, those with a birth weight of less 10% carry a 10% risk of poor perinatal outcome. [75] Interestingly, almost 50% of perinatal morbidity occurs in neonates with normal birth weights. Scott and Usher [88] found that perinatal asphyxia was more common in type II IUGR infants than type I, and Hill *et al.* [45] demonstrated that regardless of birth weight, long-term neurological morbidity was more frequent among neonates born with soft tissue wasting seen in type II growth restriction. Low *et al.* [59] have found that type II IUGR babies have an accelerated growth phase during the first 3 months post delivery, although at 12 months of age, they remain smaller than AGA babies. All IUGR infants are also prone to meconium aspiration, polycythemia, and hypoglycemia. [31] Since IUGR often occurs in mothers who are not grossly malnourished, it is likely that a subtle interplay exists between nutrients levels and inadequate placental perfusion and transfer of nutrients. [30]

The severity of growth restriction is not only related to the availability of substrate levels but is also dependent upon the maternal underlying nutritional state, the

timing, and the severity and duration of deprivation. Chronic reduction in placental perfusion and other placental diseases have been uniformally associated with smaller placental size and asymmetric fetal growth in a linear and inverse relationship. [88, 111] In longitudinal studies, changes in uterine blood flow correlate directly with changes in the growth rate of tissue, and levels of oxygen, glucose, and lactate in fetal circulation are unchanged until long after morphometric evidence of growth restriction is well established. [16-19, 100] Early adaptive measures in response to growth retardation probably include normal physiologic responses to down regulate the demand for the substrate. If the stressor continues, regional distribution of placental blood flow shunts toward the organs most essential for survival. [15] It is also thought that the placenta could respond to decreased delivery of substrates by releasing one or more growth inhibiting substances. Vascular ligation in the guinea pig resulted in identification of a protein moiety in the umbilical venous plasma of a growth retarded fetus which clearly suppresses protein synthesis in cultured hepatocytes. [49] Charlton and Johengen [14] showed that decreased fetal/placental weight induced by progressive placental emboli damage can be prevented by hyperalimentation via intravenous but not via intragastric route. Thus limited substrate availability rather than placental damage and a reduction in flow is probably the event leading to restricted growth. This study also confirms the interdependence of substrate cycling of the placenta and liver. Therefore, the data suggest that adequacy of the normal placentation process itself coupled with an autoregulated, substrate-dependent growth inhibitory response to a variety of restrictive stimuli is necessary.

In the majority of asymmetric IUGR, there is reduced maternal levels of hPL. [64] Since it reflects placental mass and not low fetal weight, low levels of this hormone can be used to identify IUGR caused by small placentas. Conversely, in multiple and diabetic pregnancies, high levels of hPL have been associated with above normal placental weight. [92] Other hormonal and metabolic changes of a growth-suppressed fetus at term include elevated catecholamine, cortisol, glucagon, beta-endorphin, essential and branched chain amino acids, and reduced prolactin, thyroxin, insulin, insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II), glucose, lactate, and triglycerides. [1, 13, 29, 90, 107]

In the U.S. more than 100,000 LGA babies are born each year with the overall incidence at 3.1–5.3%. [47] Macrosomia is associated with high maternal and perinatal morbidity and mortality. Nelson *et al.* [71] estimated that there are 164 perinatal deaths per 1000 births. The perinatal morbidity rate is at least twice that of normal sized infants and the mortality rate is at least 5 times higher. [83] This risk is even greater for those with birth weights above 4500 gm. There is also increased association with hyperglycemia, hypocalcemia, hyperbilirubinemia, polycythemia, cardiac failure, and cerebral edema from trauma or asphyxia. In addition, the condition poses increased risks of vaginal laceration, pelvic hematomas from forceps use, and uterine atony and hemorrhage to the mother. [67] Certain factors have been empirically associated with fetal macrosomia such as history of large babies, [94] multiparity, maternal obesity (greater than 15% overweight), excessive weight gain (greater than 35 pounds) during pregnancy, Class A diabetes, and post-dates delivery. [54]

There are two types of macrosomia--mechanical and metabolic. In the former, fetuses are proportional, and difficult deliveries are secondary to shoulder dystocia, which is related to increased shoulder circumference and not simply to high birth weight. [2, 53, 69] Shoulder dystocia is associated with a morbidity rate of 3.3% for babies who weigh 4100–4325 gm and 8.2% for those greater than 4500 gm. Some fetuses grow as expected during the third trimester and become macrosomic only because they do not deliver at 38 weeks and appear to continue growing. Other fetuses show growth acceleration in the third trimester and thus are macrosomic regardless of when they are delivered. [1]

Metabolic or asymmetric macrosomia is often associated with diabetes mellitus where hypertrophy and hyperplasia account for organ enlargement, which subsequently results in mechanical macrosomia and possibly death in utero. [70] Susa *et al.* [97] demonstrated hepatomegaly, cardiomegaly, and a large increase in adipose tissue deposition in the rhesus monkey associated with fetal hyperinsulinemia.

The Role of Insulin in Fetal Growth

Insulin is present in the fetus as early as nine weeks gestation [93] and increases with gestational age. The hormone is entirely of fetal origin as it does not cross the placental barrier. Only after 20 weeks gestation is the insulin response to glucose clearly manifested. [6] In addition to the placenta [77], insulin receptors have also been identified in other fetal tissue and are characterized by increased binding capacity for insulin and failure to down regulate receptor number in presence of hyperinsulinemia. [72]

Overall, diabetic patients have 15–45% incidence of producing macrosomic infants. [56] In uncontrolled diabetic mothers, fatty acid transport is increased which contributes significantly to the growth of the fetus in the third trimester resulting in a macrosomic baby. Fetal hyperinsulinemia increases the activity of insulin-sensitive tissues such as fat, muscle, and liver, resulting in increased formation of soft tissue. [51, 70] These metabolic effects often result in greater shoulder [68] and abdominal [73, 98] circumferences. Overgrowth of infants is manifested by either normal fetal length but obese or one with large body dimensions for gestational age but not obese. Fetal hyperinsulinemia is associated with increased serum IGF-I [44] whereas fetal pancreatectomy is associated with decreased levels [40]. The high insulin/glucagon ratio also promotes placental storage of triacylglycerol. During the neonatal period, 75% of infants of pregestational diabetic and 25% of infants of gestational diabetic mothers develop hypoglycemia. The infants whose metabolic state has been altered have impaired ability to react to temporary hypocaloric stress since often they are also hypoglucagonemic and have impaired glycogenolysis. Other complications include birth asphyxia, respiratory distress syndrome, cardiac dysfunction, hypocalcemia, polycythemia, jaundice, small left colon syndrome, and various congenital anomalies. [96]

There are cases however such as well controlled diabetes, where fetal insulin levels are presumably normal but fetal size is nevertheless excessive. This has lead to the speculation that other factors or peptides may also have a significant growth promoting influence. Insulin, IGF-I, and IGF-II are closely related molecules; therefore it is conceivable that IGFs may also have a significant role in fetal growth.

Insulin-like Growth Factor I (IGF-I) and Insulin-like Growth Factor II (IGF-II)

Mechanisms of control of placental and fetal growth are likely to be related and are also likely to involve the same peptides that regulate extrauterine growth, namely insulin, IGF-I, IGF-II and growth hormone (GH).

IGF-I is a basic, single chain polypeptide containing 70 amino acids with a molecular mass of 7.6 kD. IGF-II is slightly acidic consisting of 67 amino acids and weighing 7.4 kD; it has 68% homology with IGF-I, and both share with insulin structural homology, three-dimensional configuration, and the property of causing effects associated with growth hormone-dependent growth of humans. These effects include the stimulation of glucose and amino acid transport, lipid synthesis from glucose, glycogen and protein synthesis, and inhibition of lipolysis. [96, 112] Insulin, however, differs from the IGF peptides in the mechanism of transport in plasma; insulin is carried unbound while both IGF-I and IGF-II are associated with specific carrier proteins via non-covalent bonds.

Several studies have shown the presence of IGF-I and expression of its gene from rat kidney, lung, liver, and heart [26] and human fibroblasts, [20] hepatocytes, hepatic hemopoietic cells, intestine and kidney tubules, adrenal cortical cells, and skeletal and heart muscle fibers. [43] Additionally, there appear to be developmental changes in the expression of IGF-II, with increased levels of the mRNA that encodes this peptide in second trimester placentas, as compared to first trimester and term placentas. [89] In term diabetic gestations, increased placental expression of the IGF-II-specific mRNA is observed, with overlap of its levels of expression of the mRNA in diabetics and normal

controls. [89] However, relevant data are not available as to the clinical severity of the diabetes in this study, or the adequacy of glycemic control during pregnancy.

Although two somatomedins (IGF-I and IGF-II) play a role in the GH-dependent growth of humans after birth, their production during fetal life appears to be independent of GH. The fetal pituitary does not seem to be necessary for maintenance of serum IGF-I as anencephalic newborns have normal levels in cord serum. [24] It has been reported that a single ovine placental lactogen injection in the hypophysectomized rat could normalize serum IGF-I and pregnant GH-deficient women have normal IGF-I levels. [24]

Although it has not yet been proven that IGF-I stimulates fetal growth, three lines of evidence suggest that they influence fetal growth. First, IGF-I and IGF-II are capable of stimulating the proliferation of fetal cells from various species, including human. Studies have reported that IGF-I could promote proliferation of rat fibroblasts, [3] calvaria, [11] and osteoblasts, [85] chick fibroblasts, [80] and human fibroblasts [21, 106] and cartilage; [4] protein synthesis in the rat [11] and chick cartilage; [36, 42] glycogen synthesis in rat osteoblasts, [85] myoblasts, [32] and hepatocytes; [34] and differentiation of rat osteoblasts [86] and chick myoblasts. [87] IGF-II can activate proliferation of placental trophoblast. [104] Phillips *et al.* [76] have also shown that the administration of IGF-I to neonatal rats, but not GH, stimulated growth. Second, studies have demonstrated that IGFs in fetal plasma are of fetal origin since they are not transported from mother to fetus by the placenta. The liver appears to be the major source of serum IGFs in human and the peptides have been measured in extracts of many tissues. [25] In addition, IGF-I

and IGF-II have been detected in term and preterm placenta suggesting that they are involved in placental growth. [33, 65] Third, several studies have demonstrated the direct synthesis of IGFs by fetal tissues. [20, 43, 101]

The involvement of IGF-I and IGF-II in fetal growth has long been suspected, since deviant fetal growth, for example in diabetes, could not be explained by insulin levels only. Although studies have suggested that maternal serum concentrations of IGF increase during normal pregnancy, investigations of its relationship to infant size in normal pregnancy have yielded conflicting results. Multiple investigators, using a variety of assay techniques non-longitudinal studies have reported conflicting data regarding IGF-I and IGF-II levels in cord blood samples both during fetal life and at birth. [10, 35, 39, 40, 55, 82, 102, 105]

IGF Binding Proteins (IGFBP)

Binding proteins for IGF peptides have previously been purified by affinity chromatography from culture media conditioned by rat BRL cells [61] and human hepatoma cells [78] and by conventional biochemical techniques from human amniotic fluid. [28] Five classes of IGFBP have been identified in human serum; the 3 most well-characterized have been designated as IGFBP-1, IGFBP-2, and IGFBP-3. More than 98% of IGFs in normal post-natal serum is bound to IGFBP-3, a large molecular mass (150 kD) complex which is GH-dependent, thus causing circulating IGFs levels to be higher than the concentration in the tissue. This binding protein does not seem to appear until the second half of human gestation. [27] IGFBP-1 (also known as binding protein-28, placental protein 12, and pregnancy-associated endometrial α_1–globulin) is 28 kD in

size and GH-independent. It has been shown to be influenced by insulin in both normal and diabetic patients. [108] Additionally, IGFBP-1 derived from amniotic fluid has also been shown to be a cell growth inhibitor. [58] IGFBP-2, 40 kD binding protein isolated from the rat liver cell line BRL-3A, [61] has a molecular equivalent in human cerebral spinal fluid, which has a selective affinity for IGF-II. [48]

The binding of IGF-I and IGF-II to carrier proteins abolishes the insulin like properties of the free polypeptides. [52] Thus carrier proteins may also play an important role in the action of plasma IGFs and consequently may influence the regulation of fetal growth. [46, 112]

IGF Receptors

Both type 1 and type 2 receptors for IGF have been identified and partially purified from fetal tissues [81] and the placenta [89] supporting the role of IGFs in stimulating fetal growth. Type 1 is a 350 kD glycoprotein consisting of 2 extracellular α-subunits, weighing approximately 130 kD, which contain the hormone binding site and 2 transmembrane β-subunits approximately 95 kD with intrinsic tyrosine kinase activity. [62] The type 1 receptor has high affinity for IGF-I, somewhat lower and sometimes equal affinity for IGF-II, and low affinity for insulin (0.1–1.0%). Casella *et al.* [12] demonstrated that the type 1 receptor in the human placenta possess a distinct high affinity binding site for IGF-II. In fetal rat hepatocytes, Freemark *et al.* [34] showed that IGF-I was more potent than either IGF-II or insulin in stimulating glycogen synthesis, suggesting that the type 1 receptor can mediate insulin-like effects. The Type 2 receptor

is 250 kD and a monomeric transmembrane protein whose mechanism of intracellular signal transduction is unclear. It has high affinity for IGF-II, low to moderate affinity for IGF-I, and no interaction with insulin. IGF-I, like insulin, can down regulate type 1 and 2 receptors. IGF-II has not been demonstrated to have such influence.

Since IGF-I and IGF-II are thought to initiate biologic actions, which include inducing mitosis, glucose oxidation, and amino acid uptake and by binding to cell surface receptors [24, 104] and the expression of type 1 and type 2 IGF receptors also appears to be maximal during the second trimester, [89] the suggestion has been made that IGFs regulate the hyperplastic stage of fetoplacental growth, with insulin assuming a major role during the third trimester.

Purpose and Hypothesis

The objective of this investigation was to study longitudinally the relationship between normal human fetal growth and the levels of insulin, IGF-I, IGF-II, and IGFBP-3 in both the maternal and fetal compartments and to determine the presence of their receptors in placental membranes during development of the human fetus. We have hypothesized that in addition to insulin, IGF-I, IGF-II, and IGFBP-3 are involved in the regulation of fetal growth and thus their levels will vary among neonates of different birth weights in normal pregnancies.

METHODOLOGY

Subject Population. Seven patients who underwent voluntary termination of pregnancy (VTOP) by dilatation and evacuation (7–11 weeks gestation), 27 who delivered vaginally or by cesarean section (C-section) (38–41 weeks gestation), and 32 who underwent percutaneous umbilical blood sampling (PUBS) for various diagnostic purposes (21–36 weeks gestation) participated in this investigation. Patients were excluded if they did not meet the criteria for normal: absence of diabetes, elevated 1 hour glucose tolerance test, hypertension, or other complications during pregnancy. All fetal blood samples from PUBS used in this study were from fetuses who had Rh disease. However, ultrasound measurements indicated that each fetus's estimated weight and size were appropriate for gestational age. All subjects were Caucasians and ranged from 20–38 years old except for the VTOP group in which there were some were African American patients and some who were less than 18 years old. Oral consent was sought from each patient regarding collection of blood and membrane samples and of pertinent data from the medical records. This research project was approved by the Human Investigation Committee of the Yale University School of Medicine.

Index of Growth. Delivery weight was chosen as the index of fetal size. Using the gestational-weight curves established by Lubchenco *et al.*, [60] neonates were designated as AGA (between the 10th and 90th percentiles for birth weight) or LGA (> the 90th percentile for birth weight) and small or large if their weights were below or above the 50th percentile for newborns of the same age.

Medical History Collection. (Performed by author). For each fetus/neonate entered in the study, the following information was recorded from the chart: mother's age, weight and previous pregnancy history; medical history including presence of diseases which can affect fetal growth and pregnancy outcome, medications, and pregnancy complications; and fetal delivery information including gestational age, mode of delivery, sex, and birth weight and length. Gestational age was calculated in completed weeks from the first day of the mother's last menstrual period.

Serum and Amniotic Fluid Collection. (Performed by author). At the time of vaginal deliveries and C-sections, at least 2 ml of blood each was collected in a vacutainer tube containing ethylene dinitrilo tetraacetic acid (EDTA) from the mother via venipuncture and from the neonate via puncture or drainage from the umbilical cord which contained both arterial and venous blood. Fetal blood samples were obtained by direct puncture of the umbilical vessel under ultrasound guidance. At C-section deliveries, at least 2 ml of amniotic fluid was also collected. All samples were either immediately spun down at 4,000 x g for 10 minutes or placed on ice and spun within 2 hours. They were stored in aliquots of 0.5 ml at -20° C until the time of assay.

Tissue Procurement. (Performed by author). Upon delivery of the placentas from C-sections, neonatal blood was collected and the placentas placed on ice. The amnion, chorion/decidua, and placenta were isolated by visual inspection and washed with cold saline to remove adherent blood. Placentas from VTOP were immediately placed in cold

saline on ice and rinsed to remove as much blood as possible. A 2 cm x 2 cm sample of each tissue was placed in 1.5% glutaraldehyde fixative (25% glutaraldehyde [Sigma G5882], 0.04 g/ml paraformaldehyde, 0.36% sodium hydroxide, and 10% autoclaved phosphate buffered saline (PBS), which contained 8.1 mM sodium phosphate dibasic anhydrous, 1.9 mM sodium phosphate monobasic, 145 mM sodium chloride, and 1 ml 0.5 mM EDTA at pH 7.4) and stored for at least 6 hours or overnight in the dark at room temperature. This phase of tissue collection did not exceed 30 minutes. The samples were then washed twice with PBS at room temperature and put in cassettes, which were placed in 60% ethanol (ETOH) for at least 6 hours or overnight at 4°C. Tissue samples were embedded in paraffin blocks within 24 hours, sectioned (5 μm), and placed on glass microslides for immunohistochemical staining (performed by Adel Kusnitz at Yale University School of Medicine).

Immunohistochemistry

Reagents. PBS was sterilized using a millipore (0.22 μm) syringe filter and mixed with 1% bovine serum albumin (BSA). Blocking protein (human serum from normal adult volunteer) was mixed with PBS in a ratio of 1:67. The serum was collected immediately after venipuncture, stored at -20° C in aliquots, and used without refreezing. The primary antibodies (monoclonal antibody against insulin receptor, extracellular domaine, [Chemicon International, Inc., Temecula, CA] and monoclonal antibody against Type 1 IGF receptor, [Oncogene Science, Manhasset, NY]) and secondary antibody (biotinylated anti-mouse IgG (Vector, Burlingame, CA) were mixed with PBS/1% BSA

in a 1:10 ratio. These dilutions were previously titered to give an appropriate staining intensity. Streptavidin-horseradish peroxidase (Zymed Labs, Inc., So. San Francisco, CA) was prepared with PBS/1% BSA in a ratio of 1:100. The chromogen solution, diaminobenzine tetrahydrochloride - A (DABA), contained 0.045% hydrogen peroxide and 6.7% 3,3'-diaminobenzidine isopac of tetrahydrochloride [Sigma D9015] in phosphate buffer (PB), which consisted of 8.1 mM sodium phosphate dibasic anhydrous and 1.9 mM sodium phosphate monobasic at pH 7.4.

Procedure. (Performed by author). The tissue sections on the microslides were deparafinized by immersing in xylene for 7 minutes 3 times and rehydrated by sequentially dipping in 100% ETOH for 2 minutes; 75% ETOH for 2 minutes; 50% ETOH for 2 minutes; 25% ETOH for 2 minutes; double distilled water for 2 minutes; 1% hydrogen peroxide for 15 minutes; and PBS 5-6 times. The blocking solution was applied to each section and incubated in a humidified tray for 10 minutes at room temperature. The excess blocking solution was then tapped off. Next, the primary antibody solution was applied to each tissue section. The tissue was then incubated in a humidified chamber at 4° C for 20 hours. The slides were allowed to return to room temperature, and each was gently rinsed with PBS 2 to 3 times. After the secondary antibody was placed on each tissue section, all samples were incubated for 60 minutes at room temperature. The slides were then rinsed with PBS 2-3 times. The next stage consisted of applying streptavidin-horseradish peroxidase on the tissue and incubating for 30 minutes at room temperature. The slides were then rinsed with PBS and kept covered

with the buffer to keep from drying until the entire batch was completed. DABA was used to develop the tissues (approximately 5 minutes). Slides were monitored under the microscope to prevent excessive background. The peroxidase-antiperoxidase reactions were terminated by washing sections in distilled water. The tissue sections were then dehydrated by sequentially dipping in gradually increasing concentration of ETOH: 25% ETOH for 2 minutes; 50% ETOH for 2 minutes; 75% ETOH for 2 minutes; 100% ETOH for 10 minutes 2 times; and xylene for 10 minutes 2 times. Coverslips were then placed on the tissue sections using Cytoseal. Positive control consisted of identifying insulin and type 1 IGF receptors in 2 fetal liver samples (19 and 21 weeks gestation). Negative controls were done by omitting the primary antibody, the secondary antibody, streptavidin-horseradish peroxidase, or DABA and were included with each batch of slides that was stained.

Biochemical Assays

Radioimmunoassay (RIA) for insulin. Insulin concentrations were measured in maternal and neonatal sera and amniotic fluid by Aida Groszmann at Yale University School of Medicine.

Reagents. The reagents used were supplied in a commercial double antibody RIA kit (Ventrex Laboratories, Portland, ME). They included (1) assay buffer (protein stabilizer and a preservative); (2) insulin antiserum (guinea pig anti-insulin in buffer with a protein stabilizer and a preservative); (3) insulin tracer (^{125}I–insulin in

buffer with a protein stabilizer, normal guinea pig serum, and 0.1% sodium azide as a preservative); (4) precipitating agent (goat anti-guinea pig gamma-globulin and polyethylene glycol (PEG) in buffer with 0.1% sodium azide as preservative; (5) insulin standards (7 concentrations [2.5–300 μU/ml] of porcine insulin in buffer); and (6) insulin controls (lyophilized porcine insulin in human serum with 0.1% sodium azide as a preservative, which was reconstituted with distilled water).

Procedure. All standards, controls, and samples were assayed in duplicate. Three hundred μl of assay buffer were placed in 2 non-specific binding tubes, and 200 μl into 2 maximum binding tubes. Two hundred μl of each standard, control and sample were placed in the remaining tubes. Next, 100 μl of insulin tracer was added to each tube including 2 for total counts followed by 100 μl of insulin antiserum into all tubes except the total count and non-specific binding tubes. All tubes were then vortexed and incubated for 90 minutes at room temperature. One ml of precipitating agent was then added to all tubes, which were vortexed and incubated for 10 minutes at room temperature. The tubes were then centrifuged at 1,500 x g for 10 minutes at 4°C. The supernatant of each tube except the total count tubes was decanted and the rim was blotted dry. The radioactivity in all tubes was counted for 1 minute in the gamma counter. For each standard concentration, the percentage binding was calculated using the formula (counts/minutes of standard – counts/minutes of non-specific binding)/(counts/minutes of maximum binding – counts/minutes non-specific binding) x

100. The results were then plotted against the corresponding standard concentration, and the insulin concentration of test samples was read directly from the standard curve.

RIA for IGF-I. IGF-I, IGF-II, and IGFBP-3 binding protein levels were assayed by Kathy Gallego in the laboratory of Dr. E. Martin Spencer at California-Pacific Medical Center.

Separation of IGFBP-3 from IGF-I. All samples were extracted prior to RIA analysis with acid-ethanol to remove interference from endogenous binding proteins by a modification of the method of Daughaday *et al.* [23] All samples were chromatographed on the Waters C_{18} Sep Pak cartridge that was activated by passing in sequence 3 ml of acetonitrile, followed by 3 ml of distilled water, and then 3 ml of 0.1% aqueous trifluoroacetic acid (TFA). 0.2 ml of sample was acidified with 1.3 ml of 1% TFA solution, mixed and applied to the cartridge after 10 minutes at room temperature. It was possible to leave the sample in 1% TFA solution for several hours without loss of activity. Then, the entire sample was aspirated or forced through the Sep Pak slowly, 1 to 5 minutes. Subsequently, the tube containing the sample was washed with two 1 ml portions of 0.1% TFA and each applied to the cartridge in succession. After this, a final 1 ml of 0.1% TFA was used to wash the column. The washing procedure assured a quantitative transfer of the sample and removal of the dissociated carrier protein from the C_{18} cartridge. The samples were eluted using 2.2 ml of 0.1% TFA in acetonitrile. After extraction, the samples were dried by removing the acetonitrile under vacuum using a

Savant Speed Vac Concentrator (Savant Instruments, Farmingdale, NY), reconstituted in 0.5 ml of RIA buffer, and adjusted to pH 7.4 with 2 M Tris.

RIA

Reagents. The RIA buffer consisted of 30 mM phosphate containing 0.25% human serum albumin and 0.02% sodium azide at pH 7.4. The primary antibody was prepared by Reber and Liske [79]. Rabbits were immunized with human IGF-I purified by Ritschard and Roncari at Hoffman-La Roche (Basel, Switzerland) from human Cohn fraction IV. The immunization material contained a single peptide as determined by physiochemical and N-terminal microsequence analysis and performed at a level that would detect greater than 10% contamination. Its cross-reactivity with rat somatomedin-C was 35% and 3% with rat and human IGF-II. No cross-reactivity was observed with growth hormone, prolactin, insulin, glucagon, epidermal growth factor, and bradykinin. IGF-I antiserum was diluted 5,000 fold in the RIA buffer. IGF-I was iodinated using solid-phase lactoperoxidase beads according to the protocol supplied by Biorad (Richmond, CA). The labeled polypeptide was separated from the free ^{125}I by Sephadex G-50 chromatography in 50 mM Tris-HCl pH 7.4 containing 0.25% human serum albumin. The specific activity averaged 200 μCi/μg. ^{125}I-IGF-I was diluted in the RIA buffer and contained 10% Bacitracin solution. The secondary antibody was goat anti-rabbit gamma globulins (Antibodies, Inc.) titrated to 1:20 with the RIA buffer. The precipitating reagent was RIA buffer less BSA plus 6.4% PEG 8000 (Sigma).

Procedure. The RIA was set up as described above, and each sample and standard were measured in duplicate. The assay volume was 0.4 ml and contained the rabbit anti-IGF-I in a final dilution of 1:9,000 and 15,000 cpm of ^{125}I–IGF-I. The incubation was carried out at 4°C for 18 hours. The free ^{125}I–IGF-I was separated from bound by addition of rabbit gamma globulin (final concentration 1:900) and goat anti-rabbit gamma globulin (final concentration 1:120) plus PEG precipitation (final concentration 4%). After incubation for 15 minutes at 4°C, the pellet was precipitated by centrifugation at 3,000 x g for 30 minutes at 4°C. The inter-assay variation was 5.9% and intra-assay variation was 7%. Calculations of concentrations were done as described above.

RIA for IGF-II

Separation of IGFBP-3 from IGF-II. Samples were processed using a modification of the acid-ethanol procedures described above. 0.05 ml extraction buffer (8.0 M formic acid, [Fisher A-119]; 0.5% Tween-20, SurfactAmps-20, 10% solution, [Pierce 28320] and acetone [reagent grade]) was added to each 0.1 ml sample. The solution was mixed and centrifuged at 4°C for 20 minutes at 3,000 x g. The supernatant was then diluted with RIA assay buffer (50 mM sodium phosphate monobasic [Sigma S-9638], 0.1% sodium chloride [Fisher S-671], 0.1% EDTA, disodium salt [Sigma E-4884], 0.1% sodium azide [Sigma S-2002], 0.02% protamine sulfate [Sigma P-4020], and 0.05% Tween-20 [SurfactAmps-20, 10% solution, Pierce 28320] at pH 7.5) yielding a final net dilution of 1:500.

RIA

Reagents. Human IGF-II (hIGF-II) monoclonal antibody (Clone S1-F2, Amano Pharmaceutical Co., Ltd.) was reconstituted with deionized water to yield a dilution of 1:1,000. ^{125}I–hIGF-II (obtained from Mr. E. W. Schirmer, MC797) was prepared with assay buffer to give a final concentration of 500 pg/ml. Normal mouse serum (NMS) (Calbiochem 566442) was prepared by mixing it with assay buffer less protamine sulfate plus 0.2% gelatin (bovine Skin Type IV, Sigma Q-6269). The secondary antibody, goat anti-mouse gamma globulin (Calbiochem 401210) and PEG (Sigma E-4884) was mixed with assay buffer to make the precipitating reagent. Lilly Corporate Reference standards hIGF-II ([Lot RS0059] 0.05–50.00 ng/ml) were prepared by mixing with 10 mM HCl to a concentration of 1 mg/ml, verified by measuring absorbance at 276 nm.

Procedure. Standards and test samples were assayed in duplicate. The assay volume was 0.3 ml and contained the mouse anti-IGF-II in a final dilution of 1:3,000 and 15,000 cpm of ^{125}I–IGF-II. The incubation was carried out at room temperature for 24 hours. The free ^{125}I–IGF-II was separated from bound by addition of normal mouse serum and goat anti-mouse gamma globulin plus PEG precipitation. After 1 hour at room temperature, the pellet was precipitated by centrifugation at 3,000 x g for 15 minutes at 4°C. Cross-reactivity with IGF-I was less than 1%. Inter- and intra-assay variations were 8.3 and 4.5%, respectively.

RIA for IGFBP-3

Reagents. The RIA buffer used was 50 mM phosphate at pH 7.5 containing 0.1 % sodium chloride, 0.1% EDTA, 0.1% sodium azide, 0.02% protamine sulfate, and 0.05% Tween-20. The primary antibody was a polyclonal antibody prepared in rabbit host by the Laboratory of Growth and Development at California-Pacific Medical Center, San Francisco, CA. It was diluted in the RIA buffer to a titer of 1:2,667. ^{125}I–IGFBP-3 was diluted with RIA buffer to give 5 μg/1 mCi ^{125}Iodine. The secondary antibody was goat anti-rabbit gamma globulins (Antibodies, Inc.) titrated to 1:20 with the RIA buffer. The precipitating reagent used was 6.4% PEG 8000 (Sigma) in 30 mM sodium phosphate and 0.02% sodium azide at pH 7.4. Standards were prepared by serial dilution ranging from 3.4–200 ng/ml.

Procedure. All samples and standards were measured in duplicate. The assay volume was 0.3 ml and contained the rabbit anti-IGFBP-3 in a final dilution of 1:8,000 and 15,000 cpm of ^{125}I–IGFBP-3. The incubation was carried out at 4°C for 48 hours. The free ^{125}I–IGFBP-3 was separated from bound by addition of rabbit gamma globulin (final concentration 1:900) and goat anti-rabbit gamma globulin (final concentration 1:120) plus PEG precipitation (final concentration 4%). After 15 minutes at 4°C, the pellet was precipitated by centrifugation at 3,000 x g for 25 minutes at 4°C. The inter-assay variation was 7%.

Statistical Considerations. (Performed by author). Unpaired student's two-tailed t-test was used to test the significance of the difference between means. When comparing three groups 1-way analysis of variance (ANOVA) was used, and if there was a significance, Newman Keul's test was done to determine significance between individual means. Pearson's method was used to determine correlation. Multiple regression analysis was also used to determine the best fit curve. All results are reported as the mean (M) $\pm$ the standard error of the mean (SEM). P value of < 0.05 was considered significant.

RESULTS

The Relationship of IGF-I, IGF-II, and IGFBP-3 to Gestational Age

In **Table 1**, fetal serum IGF-I, IGF-II, and IGFBP-3 levels remained stable between 21–36 weeks gestation in all fetuses. At term, large neonates showed a significant increase in IGF-I levels (53 ± 8 ng/ml and 58 ± 7 ng/ml vs. 86 ± 6 ng/ml, $p<0.05$). AGA neonatal IGF-I levels were significantly higher only when compared to the 21–27 weeks gestation group and not the 28–36 weeks gestation group. Although not statistically significant, fetal IGF-I levels in the LGA group showed a similar trend. Fetal IGF-I levels in the small group did not show a significant change from levels measured during 21–36 weeks gestation.

Using ANOVA, comparison of IGF-II levels between late second trimester (21-27 weeks), early third trimester (28–36 weeks), and each of the term groups was not statistically significant. However, comparison between two means revealed that IGF-II levels in the term small group was significantly lower than levels measured during 28–36 weeks gestation (128 ± 30 ng/ml vs. 229 ± 27 ng/ml, $p<0.05$). The student's t-test also showed that in the term large group, IGF-II levels were significantly elevated compared to levels in the 21–27 weeks gestation group (219 ± 11 ng/ml vs. 177 ± 15 ng/ml, $p<0.05$). IGF-II levels in the AGA and LGA groups were similar to levels measured during 21–36 weeks gestation.

Both LGA and large neonates also had increased IGFBP-3 levels at term (0.80 ± 0.08 μg/ml and 0.80 ± 0.05 μg/ml vs. 1.10 ± 0.07 μg/ml, $p<0.05$; 0.80 ± 0.08 μg/ml and 0.80 ± 0.05 μg/ml vs. 1.10 ± 0.04 μg/ml, $p<0.05$, respectively). IGFBP-3 levels

in the AGA and small groups were not statistically different from levels during 21–36 weeks gestation.

In Figures 1 and 2, gestational age positively correlated with IGF-I and IGFBP-3 levels ($r = +0.297$, $p < 0.05$ and $r = +0.386$, $p < 0.01$, respectively). IGF-II levels did not correlate with gestational age.

Figure 3 shows that during mid and late gestation, fetal serum IGF-I and IGF-II levels directly correlated with fetal serum IGFBP-3 levels ($r = +0.692$, $p < 0.001$ and $r = +0.458$, $p < 0.002$, respectively).

The Relationship of Insulin, IGF-I, IGF-II, and IGFBP-3 to Neonatal Weight

There is a direct and positive correlation between neonatal serum IGF-I and neonatal serum IGFBP-3 levels and birth weight ($r = +0.564$, $p < 0.02$ and $r = +0.503$, $p < 0.05$, respectively) in the AGA group **(Figures 4 and 5)**. Correlation analysis did not demonstrate a similar relationship between neonatal IGF-II and birth weight in this group. Within the LGA, small, and large groups, there was also no correlation between birth weight and IGFs or binding protein levels. There was no relationship between serum insulin levels and birth weight in any group.

When the neonates were classified as AGA and LGA, there was no significant difference in neonatal serum insulin, IGF-I, IGF-II or IGFBP-3 levels between the two groups (**Table 2**). However, when divided into small and large neonates, neonatal serum IGF-I (40 ± 11 ng/ml vs. 86 ± 6 ng/ml, $p < 0.01$), IGF-II (128 ± 30 ng/ml vs. 219 ± 11 ng/ml, $p < 0.01$), and IGFBP-3 levels (0.7 ± 0.25 μg/ml vs. 1.1 ± 0.04 μg/ml,

$p < 0.01$) were significantly higher in the large neonates. The insulin levels were comparable between these two groups **(Figures 6 and 7)**.

Maternal Serum Insulin, IGF-I, IGF-II, and IGFBP-3 Levels

Table 3 shows maternal serum insulin, IGF-I, IGF-II, and IGFBP-3 levels during early (trimester 1) and late pregnancy (term). At term, maternal serum IGF-I and IGFBP-3 levels were significantly higher than maternal levels during early pregnancy. This finding was not observed with insulin or IGF-II levels in any of the 4 term groups. In **Figure 8**, in the AGA group, maternal serum IGF-I levels were found to increase along with maternal serum IGFBP-3 levels. Maternal serum IGF-II levels did not correlate with IGFBP-3 levels in this group. In **Figure 9**, similarly in the large group, maternal IGF-I levels directly correlated with maternal IGFBP-3 levels. Again, there was no relationship between maternal IGF-II and IGFBP-3 levels. There also was no correlation between maternal serum IGFs and IGFBP-3 levels within the LGA and small groups.

Table 4 shows that there was no significant difference in maternal peptides levels between AGA and LGA neonates. Maternal peptides levels between small and large groups also did not differ significantly **(Table 5)**.

The Relationship Between Maternal and Neonatal Insulin, IGF-I, IGF-II and IGFBP-3 Levels

Figures 10 and 11 compare maternal and neonatal peptides levels within the LGA and AGA groups. Within each group, maternal serum levels of IGF-I, IGF-II, and IGFBP-3 were significantly higher than the neonatal levels. There was a significant difference between maternal and neonatal levels of insulin in the AGA group but not the LGA group.

Figures 12 and 13 also compare peptides levels in maternal and neonatal compartments but within small and large neonates. Again, maternal serum levels of IGF-I, IGF-II, and IGFBP-3 were significantly higher than neonatal levels. Similarly, in the large group, but not the small group, maternal serum levels of insulin were significantly higher than neonatal levels.

Amniotic Fluid IGF-I, IGF-II, and IGFBP-3 Levels

Table 6 shows the comparison of maternal, neonatal, and amniotic fluid peptides levels of AGA term neonates. Neonatal serum IGF-I, IGF-II, and IGFBP-3 levels were similar to amniotic fluid levels (105 $\pm$ 6 ng/ml vs. 124 $\pm$ 41 ng/ml; 215 $\pm$ 27 ng/ml vs. 299 $\pm$ 81 ng/ml; 1.10 $\pm$ 0.08 μg/ml vs. 1.80 $\pm$ 0.30 μg/ml, respectively), and both were lower than maternal levels (343 $\pm$ 55 ng/ml, $p<0.01$; 510 $\pm$ 45 ng/ml, $p<0.01$; 3.90 $\pm$ 0.20 μg/ml, $p<0.01$, respectively).

Insulin and Type 1 IGF Receptors in Placental Membranes

Insulin and type 1 IGF receptors were semi-quantitatively assayed using immunohistochemical techniques. Four different intensities of staining were observed, and results were reported as trace positive, slight positive, moderately positive, and strongly positive. Type 2 IGF receptors were not assayed because antibodies were not commercially available.

Figures 14–17 show the negative controls of insulin receptor staining. The stained tissue were red blood cells. **Figures 18–21** are representative of the insulin receptors stained in the first trimester placenta and in the amnion, chorion/decidua, and placental tissue of term AGA placenta. All (7/7) of the first trimester placenta, term amnion tissue, and term chorion/decidua tissue stained strongly positive. Although also present in 100% of term placental tissue, the receptors stained only moderately positive.

Figures 22–25 show negative control for type 1 IGF receptor staining. The stained tissue were red blood cells. **Figures 26–29** are representative of the type 1 IGF receptors identified in first trimester placenta and term placental tissues. All (7/7) of the first trimester placenta stained strongly positive for type 1 IGF receptors. While 100% of term amnion did contain type 1 IGF receptors, they were only moderately stained. Type 1 IGF receptors were detected in only 56% (4/7) of the chorion/decidua tissue, and all were trace positive. In placental membranes, type 1 IGF receptors were identified in all samples as slight positive staining.

DISCUSSION

Our investigation produced the following findings: (1) fetal IGF-I, IGF-II, and IGFBP-3 remained stable between 21–36 weeks; (2) fetal serum IGF-I and IGFBP-3 correlated directly with fetal age and birth weight; (3) large neonates had higher IGF-I, IGF-II, and IGFBP-3 levels than small neonates; (4) maternal IGF-I levels correlated directly with maternal IGFBP-3 levels, and both were significantly higher at delivery than during first trimester; (5) at term, maternal serum levels of IGF-I, IGF-II, and IGFBP-3 were significantly higher than both neonatal and amniotic levels; (6) insulin was also present in both maternal and fetal sera but was not associated with birth weight in normal neonates; and (7) insulin and type 1 IGF receptors were detectable in placental membranes as early as 7 weeks gestation and were present in the amnion, chorion/decidua, and placental tissues at term.

IGF-I and IGF-II elicit classical insulin effects on all target tissues of insulin and have been shown to be potent growth promoting stimuli for many different cultured cells in vitro. [3, 11, 32, 34, 35, 80, 85, 104] Moreover, many clinical and animal studies point to the relationship between growth and IGF-I levels. The question of whether or not IGFs also have growth promoting effects of fetal growth in utero has not been definitively determined.

IGF-I and IGF-II levels have previously been measured in maternal serum and in cord blood in cross-sectional studies which have yielded conflicting results. [10, 35, 39, 40, 55, 82, 102, 105] We studied changes in insulin, IGF-I, IGF-II, IGFBP-3 levels as well as insulin and type 1 IGF receptors throughout pregnancy, in both maternal and fetal

compartments. We used a highly specific RIA in an attempt to resolve this conflict surrounding the growth promoting effect of IGF in the fetus.

In the present study we demonstrated that fetal IGF-I, IGF-II, and IGFBP-3 remained stable between 21–36 weeks. Our longitudinal approach established that in large neonates, significant increases of IGF-I occurred in fetal serum but not until 36 weeks gestation. Although we also expected LGA neonates to have a significant elevation in IGF-I levels, we only observed a rise that was not statistically significant. This finding perhaps was due to the small number of subjects in the LGA group. Conversely, while we did not expect a significant difference in IGF-I levels in the AGA group, we found that they were significantly higher when compared to levels measured in the 21–27 weeks gestation group. Since the levels were not significantly different from levels from the 28–36 weeks gestation group, this observation most likely was due to the fact some of the neonates in the large group also qualified to be included in the AGA group.

We were able to show that IGF-I levels had a positive relationship with gestational age during late second trimester through term when we used correlation analysis even though they did not change significantly between 21–36 weeks gestation by unit analysis. Perhaps the increment of elevation over time is small such that correlation analysis will be able to show a significant trend since it examines a continual change and unit analysis will not be able detect such a change. In addition to previous studies that have demonstrated this finding, [10, 39, 41] Lassarre *et al.* [55] found a positive correlation between

serum levels of both IGF-I and IGF-II and hPL after 33 weeks gestation, suggesting that hPL may have a regulatory role in IGFs synthesis by the human fetal liver.

The comparison of IGF-II levels during late second trimester, early third trimester, and at term (all four groups) by ANOVA was not statistically significantly. However, since the raw data suggested that there was clinical significance, we compared IGF-II levels in the term small neonates and term large neonates with levels during 21–27 weeks gestation and 28–36 weeks gestation and found that IGF-II levels in small neonates were significantly lower than the 28–36 weeks gestation group, and IGF-II levels in the large neonates were significantly higher than the 21–27 weeks group. We expected to but failed to find a significant difference when comparing IGF-II levels in the small group to the late second trimester group, which may suggests that IGF-II levels are reduced at term but only in small neonates and that the relative reduction is small. Nevertheless, because we were not able to establish a best fit curve between gestational age or birth weight and IGF-II levels, the lack of significance when compared to late second trimester is may be related to the small number of subjects in the small group. Our only explanation for why IGF-II levels in the large neonates were significantly higher than levels in the 21–27 weeks gestation group but were not significant when comparing between late second trimester and early third trimester is that the variance in the early trimester group was unusually large. While several previous studies also did not identify a relationship between gestational age and fetal IGF-II, [5, 41, 110] Bennett *et al.* [10] did demonstrate that fetal age correlated with IGF-II levels in normal term and

preterm infants. These data indicate that the relationship between fetal IGF-II levels and the duration of gestation needs to be further clarified in a larger study.

Our study, however, did show that IGF-I, IGF-II, and IGFBP-3 all exhibited similar trends during mid and late gestation. Therefore, it seems conceivable that, although both peptides and the binding protein may interplay to regulate fetal growth, IGF-II is not the dominant hormone influencing growth. It can also be postulated that by measuring the circulating levels of IGF-I and IGF-II we evaluated only the endocrine action of these peptides on intrauterine growth and not their possible paracrine action.

Conflicting information have been reported regarding the relationship between IGF levels and neonatal birth weight, which is partly due to the different criteria used to divide study subjects and partly due to the use of many different assay techniques. We chose to divide the neonates using two different criteria in order to properly analyze the data in comparison to previously studies.

When we classified our neonatal subjects as small or large, we found that large neonates had significantly higher levels of IGF-I, IGF-II, and IGFBP-3 than small neonates. Our findings agree with a recent study conducted by Lassarre *et al.* [55] Using the same classification, they studied IGF-I, IGF-II and their binding protein in the fetal circulation in both normal and intrauterine growth retarded fetuses and found a significant association between IGF-I levels and fetal weight. While many reports have also shown a positive correlation between IGF-I levels at term and birth weight, [5, 7, 10, 24, 41, 50, 102] Wang *et al.* [105] measured IGF-I and IGFBP-3 in the maternal circulation as well as in cord blood of SGA and AGA neonates and found an inverse correlation between IGF-I

levels in the umbilical artery and vein and birth weight. The authors concluded that while it is very likely that fetal growth is influenced by the interaction between IGF-I, IGFBP-3, and their receptors at the local tissue levels, the circulating concentration of IGF-I and IGFBP-3 may only indirectly reflect their activity.

While there was a positive correlation between fetal IGF-I levels and birth weight, no such relationship existed with IGF-II in our study. Bennett *et al.*, [10] however, reported that IGF-II correlated with birth weight, but only in term and not preterm infants. Since they also used specific RIAs to measure IGFs levels, their method of dividing the study subjects (excluding all neonates whose weights were greater or less than two standard deviations from the mean) is the only factor that can account for this contrast, although it is unlikely. Our finding that insulin levels were comparable in all neonates regardless of birth weight further strengthens our hypothesis that IGF-I has an important role in the control of intrauterine growth.

In this investigation we measured only IGFBP-3 levels. Our data showed that during fetal life, IGFBP-3 production is adapted to the increase the bioavailability to IGF-I synthesis. We have also determined that IGFBP-3 levels correlated with gestational age and birth weight and were significantly higher in large neonates when compared to small neonates. Since it has been demonstrated that binding of proteins to IGFs abolishes their activity, [46] it is plausible that in large neonates or fetuses with above normal acceleration of growth at term, one of the mechanisms to control the growth rate is to decrease the biologically active IGF concentration via increasing production of

IGFBP-3. Our finding therefore supports the role of binding proteins in the regulation of fetal growth.

Maternal serum IGFBP-3 levels also rose during the third trimester, concomitant with increasing maternal IGF-I levels, indicating that there is an association between IGF-I and IGFBP-3. Despite similar observations with fetal serum IGF-I and IGFBP-3 levels, it is very unlikely that there is placental transfer of the peptides as both are greater than 1000 daltons. [66, 99] Moreover, in all neonates, maternal levels of IGF-I, IGF-II, and IGFBP-3 were significantly higher than neonatal levels. This implies that IGF-I production in the maternal and fetal compartments are independent of each other. These results are in agreement with previous studies. [7, 8, 38, 84]

In the maternal compartment, our study showed that IGF-I and IGFBP-3 were significantly higher at delivery than during first trimester, but there was no association between maternal IGF-I and IGF-II levels and neonatal birth weight. Our results agree with previous studies [37, 108, 110] that maternal IGF-I concentration correlated significantly with the duration of gestation but not with neonatal birth weight. These data, along with the unlikelihood that there is placental transfer of IGFs, suggest that maternal IGF-I does not have a direct role in fetal growth. Wilson *et al.* [110] found that maternal IGF-II during the third trimester was significantly higher than the first trimester. Our results conflict and we cannot offer an explanation for this discrepancy as IGFs levels were measured using the same techniques in both studies. However, since both Furlanetto *et al.* [37] and Wilson *et al.* [110] demonstrated that during the post partum period,

there was a striking decline in maternal IGF-I and IGF-II, it is possible that maternal IGFs primarily regulate placental growth which indirectly affects fetal growth.

We demonstrated that fetal and amniotic fluid levels of IGFs and IGFBP-3 were similar, suggesting that exchange can exist between the two compartments. Merimee *et al.* [63] studied IGF-I, IGF-II, IGFBP-1, and IGFBP-3 in amniotic fluid and found that while IGF-I concentration was constant throughout gestation, IGF-II decreased precipitously during late gestation. The total binding protein levels decreased significantly during mid gestation and remained constant during the last period of gestation. The authors concluded that the distinct pattern of IGF-II and IGFBPs variation suggests a dynamic control of IGF in the amniotic fluid during normal pregnancy. Since Fant *et al.* [33] reported that binding proteins were produced by preterm but not term placenta and Povoa *et al.* [78] demonstrated that IGFBP isolated from a human hepatoma cell line was identical to the human amniotic fluid IGFBP, it is possible that during early gestation, the placenta, in addition to fetal liver, serves as a source of IGFs and binding protein. Amniotic fluid then functions as a reservoir for IGF-I, IGF-II, and IGFBP-3 throughout gestation should it become necessary for the fetus to modulate growth in response to stressors.

Our study showed that maternal serum insulin levels did not differ significantly between any of the four term groups and neither maternal nor fetal/neonatal insulin levels correlated with birth weight in normal neonates. These findings suggest that in normal pregnancy, insulin does not have the primary role in regulating fetal growth. When we compared maternal and neonatal sera insulin levels, we found that maternal levels were

significantly higher only in the AGA and large groups. Again, we attribute these observations to the fact that the AGA and large groups included some of the same study subjects. Although maternal levels are higher than neonatal levels in two of the four groups, we believe that this is not relevant in the control of fetal growth because it is well accepted that insulin does not cross the placental barrier and all of our subjects are non-diabetic.

Insulin and type 1 IGF receptors were detected in all first trimester placental membranes as early as 7 weeks gestation and in the amnion, chorion/decidua, and placental tissues of term placentas. Type 1 IGF receptors stained more strongly in first trimester placentas than in term placentas while insulin receptors stained relatively equally throughout. These results may reflect a down regulation of the type 1 IGF receptors in the presence of high IGF-I levels and conversely lack of down regulation of the insulin receptors in the presence of stable insulin levels.

In conclusion, the data presented in this study demonstrated the bioavailability of other in utero fetal growth promoters (IGF-I and IGF-II), their binding protein (IGFBP-3), and their receptor (type 1 IGF) during mid and late gestation. We showed that fetal levels of IGF-I and IGFBP-3 were associated with gestational age and fetal birth weight and demonstrated that large neonates had significantly higher levels of IGFs and IGFBP-3 than small neonates at birth in normal pregnancies. We also established that maternal and fetal IGF-I, IGF-II, and IGFBP-3 are produced and most likely act independently of each other. Whether or not fetal IGF-I in conjunction with fetal IGFBP-3 and IGF-II can account for the excessive growth seen in diabetes or whether

it can be used to treat fetal growth reduction remain unknown and will require further study.

TABLES AND FIGURES

Gestational Age (Weeks)	Fetal IGF-I (ng/ml)	Fetal IGF-II (ng/ml)	Fetal IGFBP-3 (μg/ml)
21–27 (n=13)	53 ± 8	177 ± 15	0.80 ± 0.08
28–36 (n=19)	58 ± 7	229 ± 27	0.80 ± 0.05
38–41 / AGA (n=18)	79 ± 8*	208 ± 17	0.96 ± 0.05
38–41 / LGA	77 ± 12 (n=8)	199 ± 8 (n=8)	1.10 ± 0.07*+ (n=9)
38–41 / Small (n=4)	40 ± 11	128 ± 30+	0.70 ± 0.25
38–41 / Large	86 ± 6*+ (n=22)	219 ± 11* (n=22)	1.10 ± 0.04*+ (n=23)

TABLE 1: Fetal Serum IGF-I, IGF-II, and IGFBP-3 Levels During Pregnancy Mean ± SEM; * $p<0.05$ vs. Gestational Age 21–27 Weeks; + $p<0.05$ vs. Gestational Age 28–36 Weeks; In the LGA and Large Groups, 1 data sample for IGF-I and IGF-II was not available due to technical difficulty with the assay and subsequently insufficient serum.

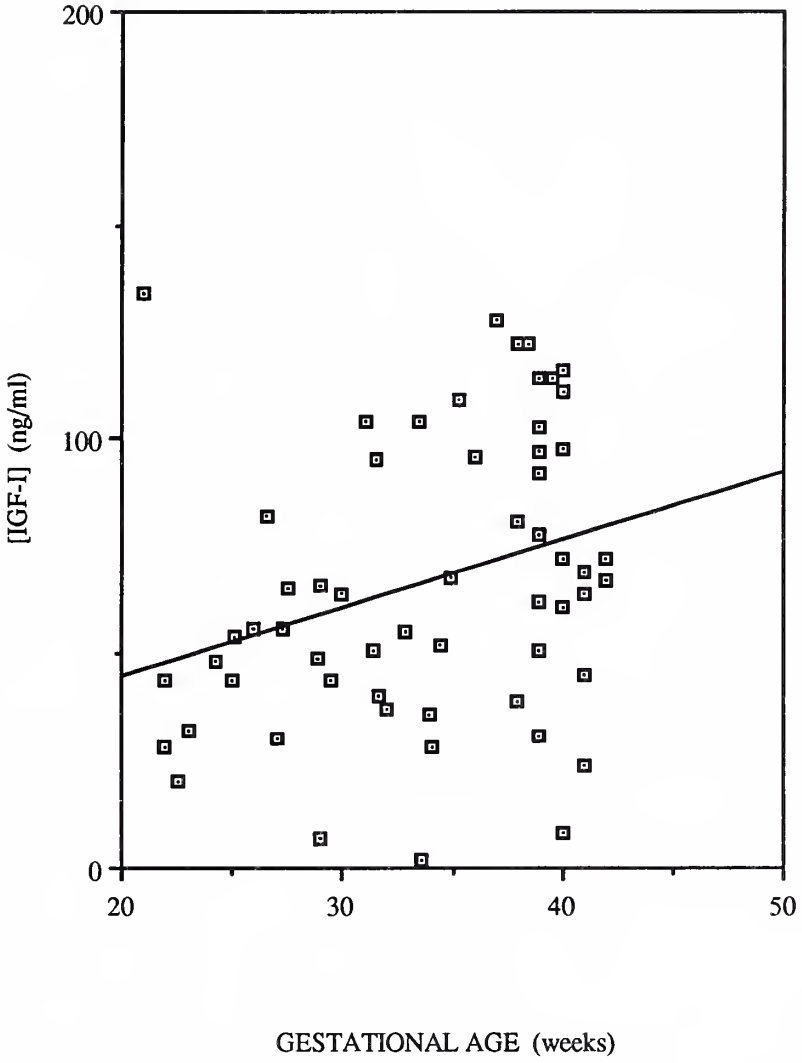

FIGURE 1: Relationship Between Fetal Serum IGF-I Levels and Gestational Age
$n = 58$; $r = + 0.297$; $p < 0.05$

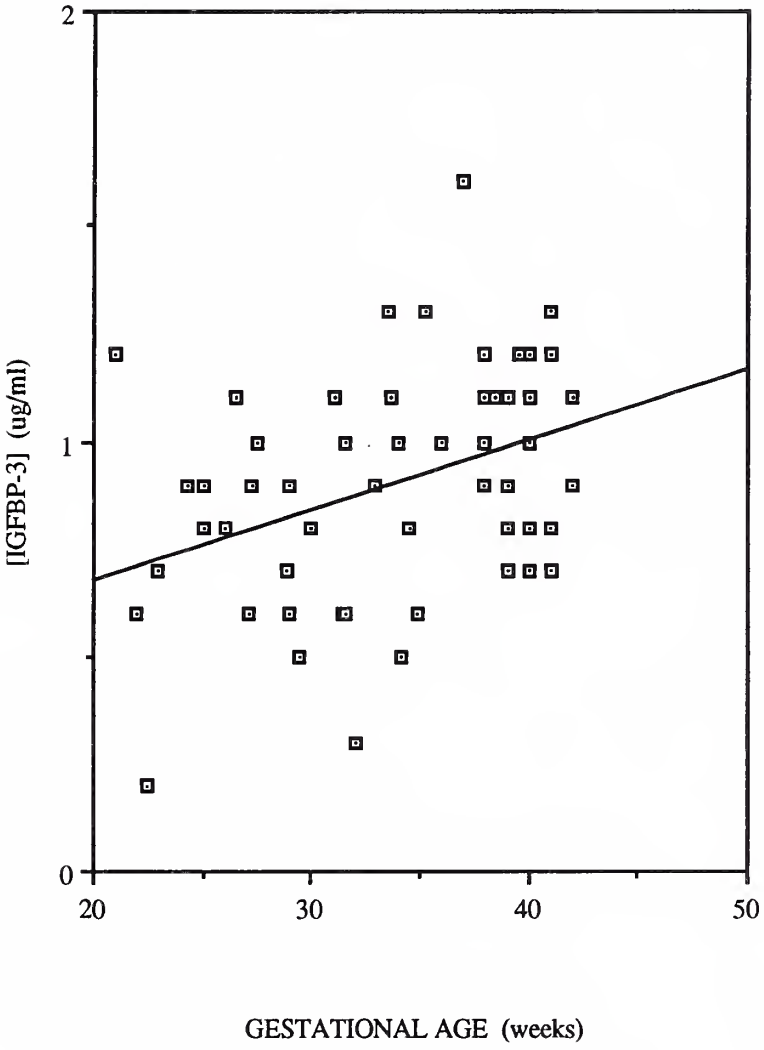

FIGURE 2: Relationship Between Fetal Serum IGFBP-3 Levels and Gestational Age
$n = 59$; $r = +0.386$; $p < 0.01$

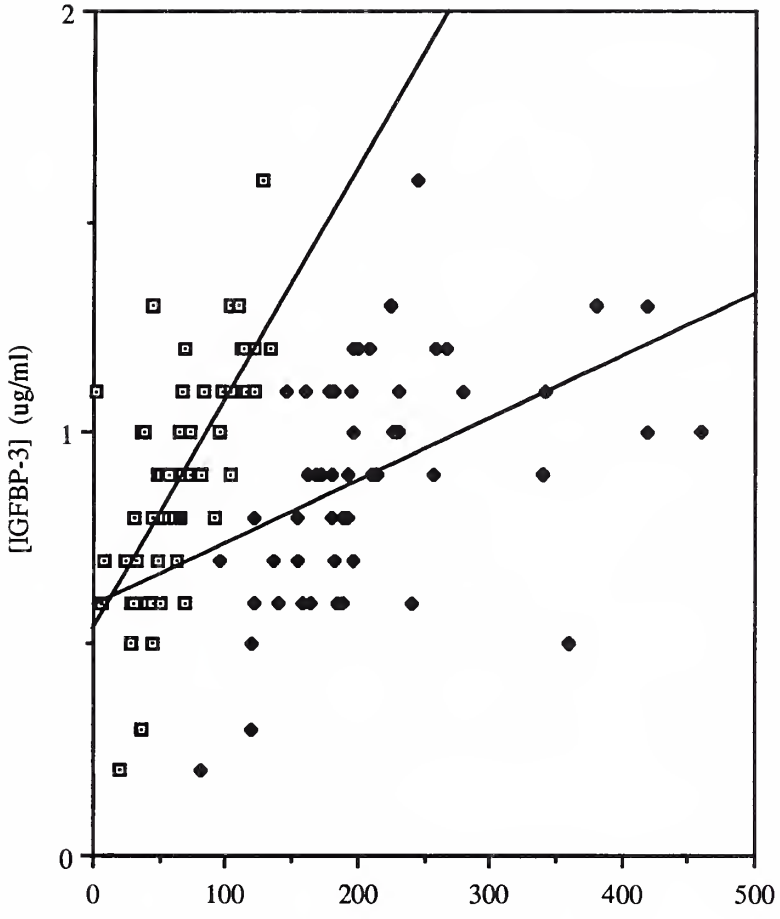

FIGURE 3: Relationship Between Fetal Serum IGF-I and IGF-II Levels and Fetal Serum IGFBP-3 Levels During Mid and Late Gestation

- ▫ IGF-I $n = 58$; $r = +0.692$; $p < 0.001$
- ◆ IGF-II $n = 58$; $r = +0.458$; $p < 0.002$

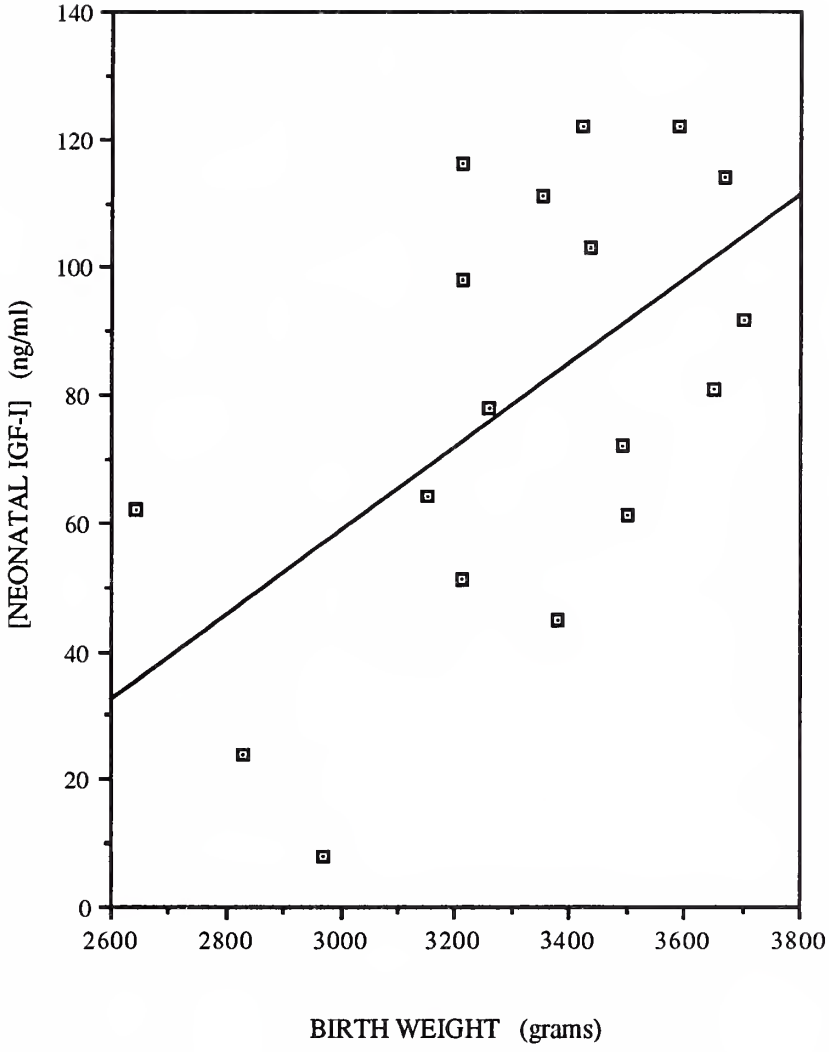

FIGURE 4: Relationship Between Neonatal Serum IGF-I Levels and Birth Weight in AGA Group
$n = 18$; $r = +0.564$; $p < 0.02$

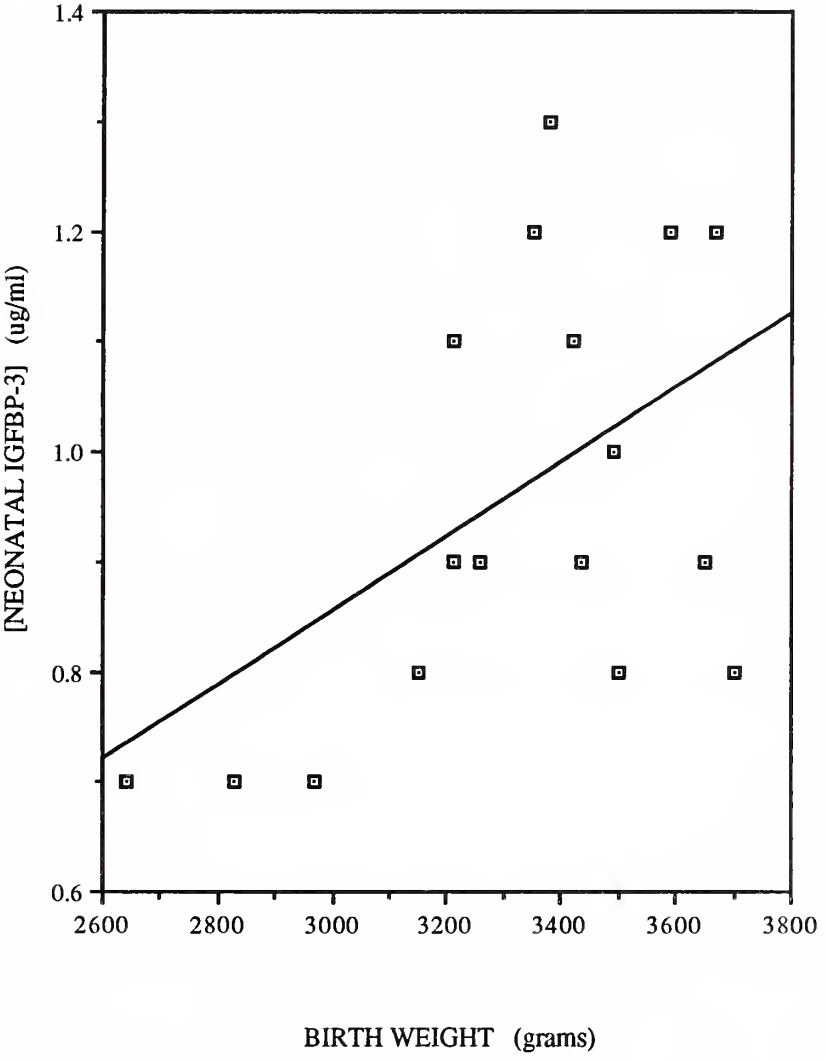

FIGURE 5: Relationship Between Neonatal Serum IGFBP-3 Levels and Birth Weight in AGA Group
$n = 18$; $r = + 0.503$; $p < 0.05$

	AGA (n=18)	LGA (n=9)	p Value
Gestational Age (weeks)	40 ± 0	40 ± 1	--
Weight (grams)	3315 ± 68	4294 ± 120	--
Neonatal Insulin (μU/ml)	16 ± 1	18 ± 5 (n=8)	NS
Neonatal IGF-I (ng/ml)	79 ± 8	77 ± 12 (n=8)	NS
Neonatal IGF-II (ng/ml)	208 ± 17	199 ± 8 (n=8)	NS
Neonatal IGFBP-3 (μg/ml)	0.96 ± 0.05	1.10 ± 0.07	NS

TABLE 2: Neonatal Serum Insulin, IGF-I, IGF-II, and IGFBP-3 Levels in AGA and LGA Groups
Mean ± SEM; NS = not significant

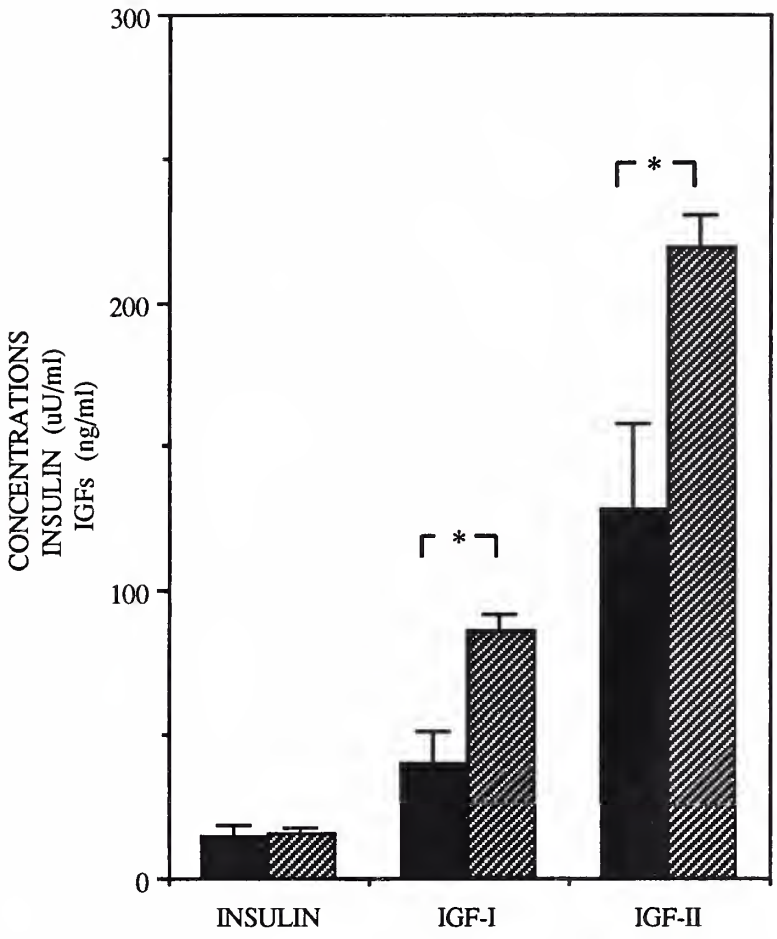

FIGURE 6: Neonatal Serum Insulin, IGF-I, and IGF-II Levels in Small and Large Neonates

■ SMALL (n =4)

▨ LARGE (n=22)

* $p < 0.01$

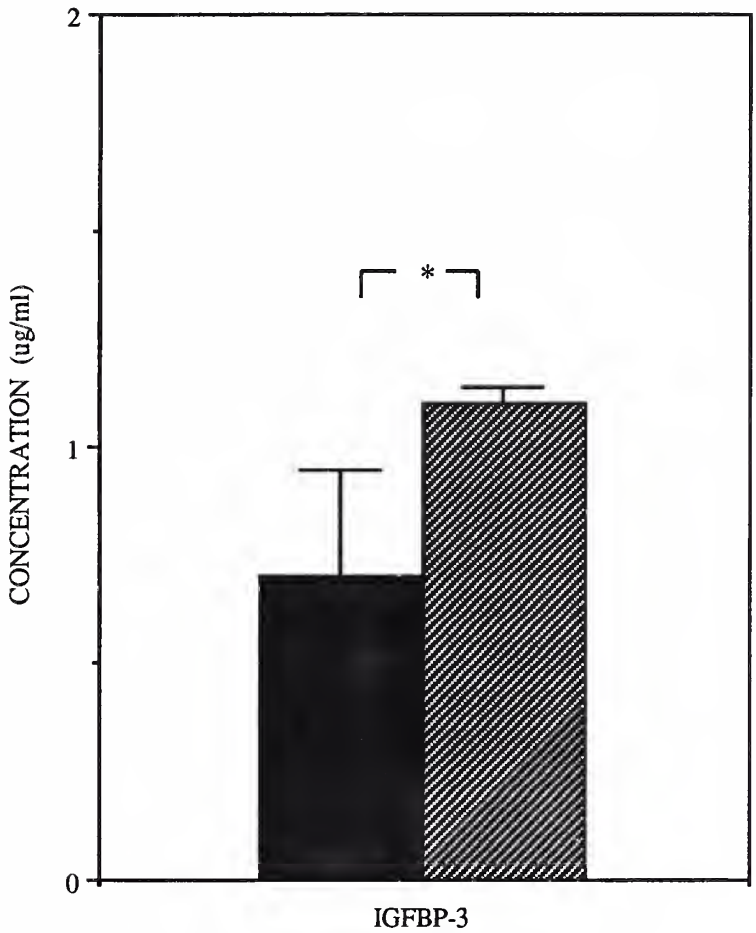

FIGURE 7: Neonatal Serum IGFBP-3 Levels in Small and Large Neonates

■ SMALL (n=4)

▨ LARGE (n=23)

* $p < 0.001$

	Maternal Insulin (μU/ml)	Maternal IGF-I (ng/ml)	Maternal IGF-II (ng/ml)	Maternal IGFBP-3 (μg/ml)
Trimester 1 (n=7)	22 ± 4	145 ± 15	535 ± 33	2.50 ± 0.20
Term / AGA (n=18)	31 ± 5	387 ± 35*	602 ± 32	3.60 ± 0.10*
Term / LGA (n=9)	28 ± 7	391 ± 28*	583 ± 59	3.50 ± 0.10*
Term / Small (n=4)	26 ± 5	412 ± 86^{+}	622 ± 101	3.30 ± 0.30$^{\#}$
Term / Large (n=23)	30 ± 5	384 ± 26*	591 ± 30	3.60 ± 0.10*

TABLE 3: Maternal Serum Insulin, IGF-I, IGF-II, and IGFBP-3 Levels During Early (Trimester 1) and Late Pregnancy (Term)
Mean ± SEM; * $p<0.001$ vs. Trimester 1; + $p<0.02$ vs. Trimester 1; # $p<0.05$ vs. Trimester 1

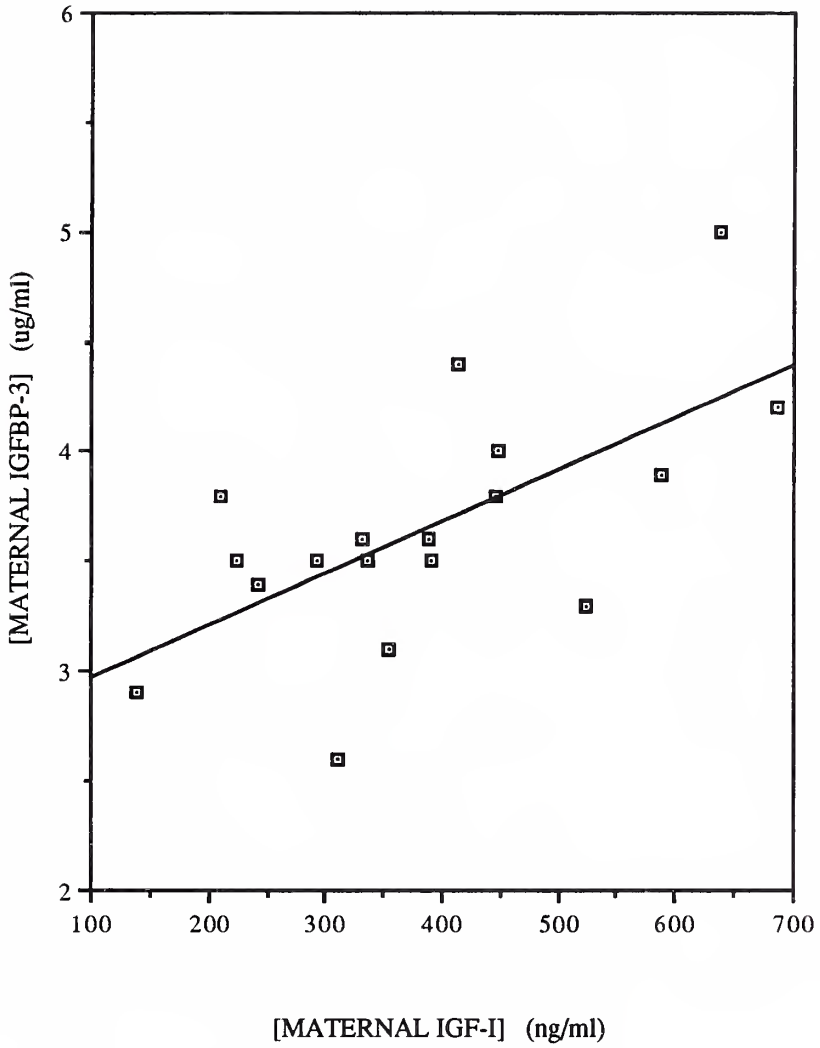

FIGURE 8: Relationship Between Maternal Serum IGF-I and IGFBP-3 Levels in AGA Group
n = 18; r = + 0.640; $p < 0.01$

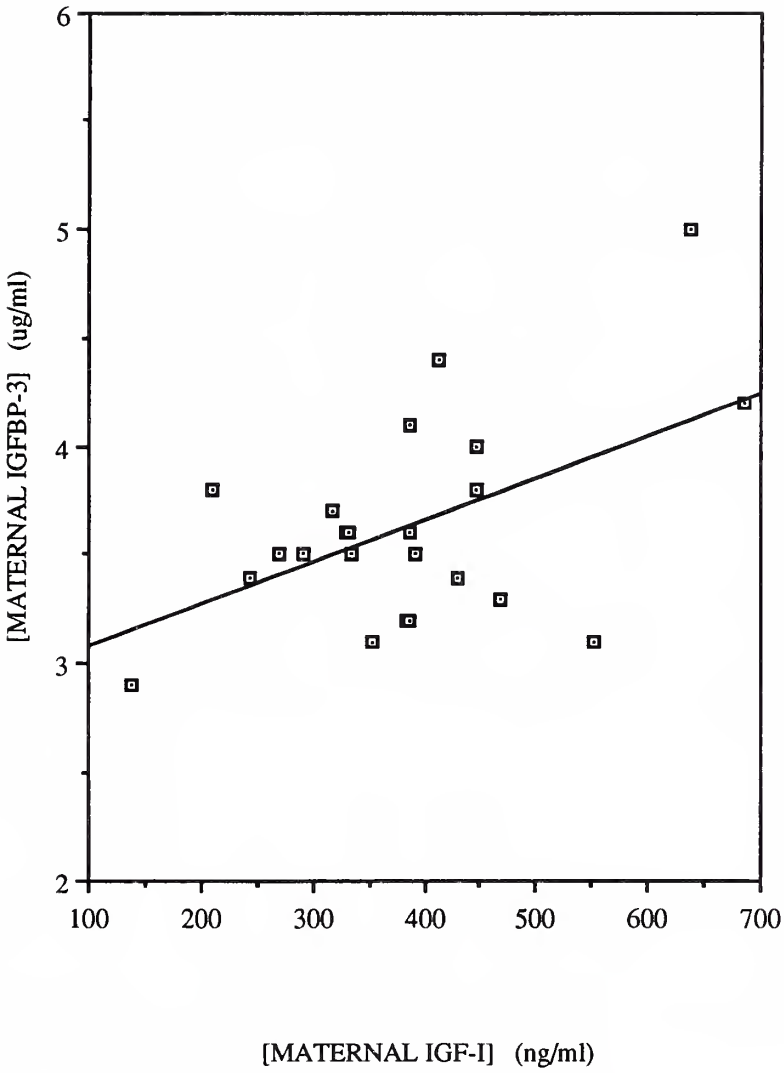

FIGURE 9: Relationship Between Maternal Serum IGF-I and IGFBP-3 Levels in Large Group
n = 23; r = +0.518; p < 0.02

	AGA (n=18)	LGA (n=9)	p Value
Gestational Age (weeks)	40 ± 0	40 ± 1	--
Weight (grams)	3315 ± 68	4294 ± 120	--
Maternal Insulin (μU/ml)	31 ± 5	28 ± 7	NS
Maternal IGF-I (ng/ml)	387 ± 35	391 ± 28	NS
Maternal IGF-II (ng/ml)	602 ± 32	583 ± 59	NS
Maternal IGFBP-3 (μg/ml)	3.60 ± 0.10	3.50 ± 0.10	NS

TABLE 4: Maternal Serum Insulin, IGF-I, IGF-II, and IGFBP-3 Levels in AGA and LGA Groups
Mean ± SEM; NS = not significant

	SMALL (n=4)	LARGE (n=23)	p Value
Gestational Age (weeks)	40 ± 0	38 ± 2	--
Weight (grams)	2898 ± 108	3770 ± 104	--
Maternal Insulin (μU/ml)	26 ± 5	30 ± 5	NS
Maternal IGF-I (ng/ml)	412 ± 86	384 ± 26	NS
Maternal IGF-II (ng/ml)	622 ± 101	591 ± 30	NS
Maternal IGFBP-3 (μg/ml)	3.30 ± 0.30	3.60 ± 0.10	NS

TABLE 5: Maternal Serum Insulin, IGF-I, IGF-II, and IGFBP-3 Levels in Small and Large Groups
Mean ± SEM; NS = not significant

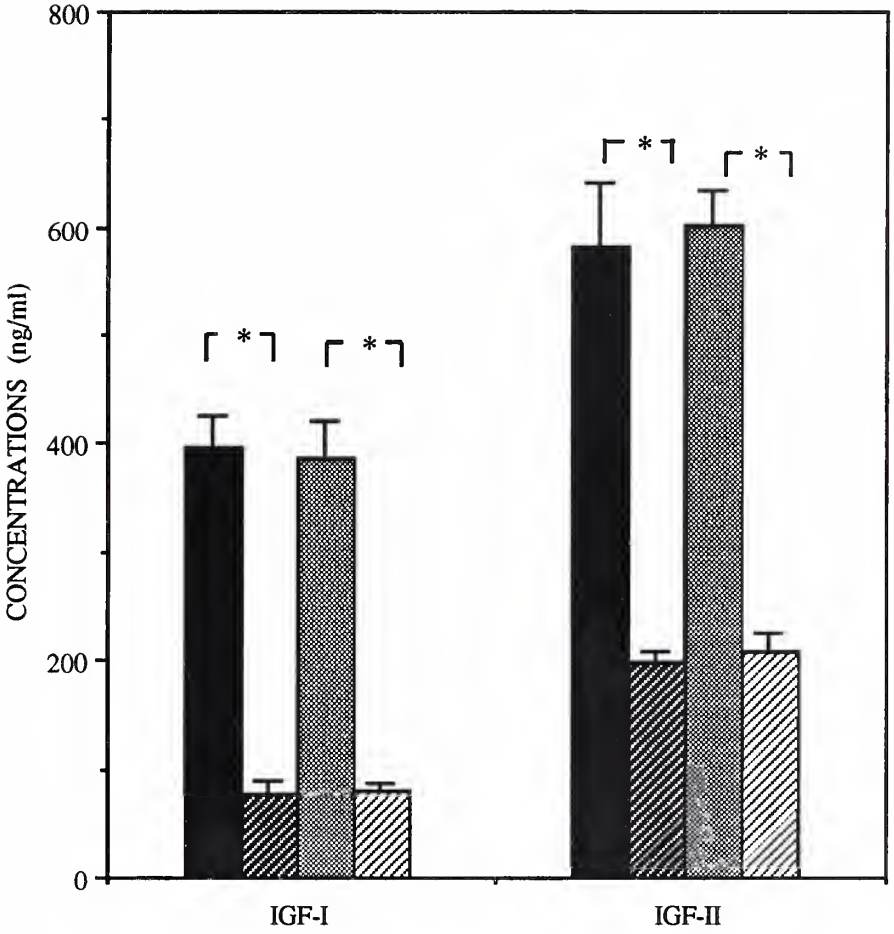

FIGURE 10: Comparison of Maternal and Neonatal IGF-I and IGF-II Levels within LGA and AGA Neonates

LGA - MATERNAL (n = 9)
LGA - NEONATAL (n = 8)
AGA - MATERNAL (n = 18)
AGA - NEONATAL (n = 18)

* $p < 0.001$

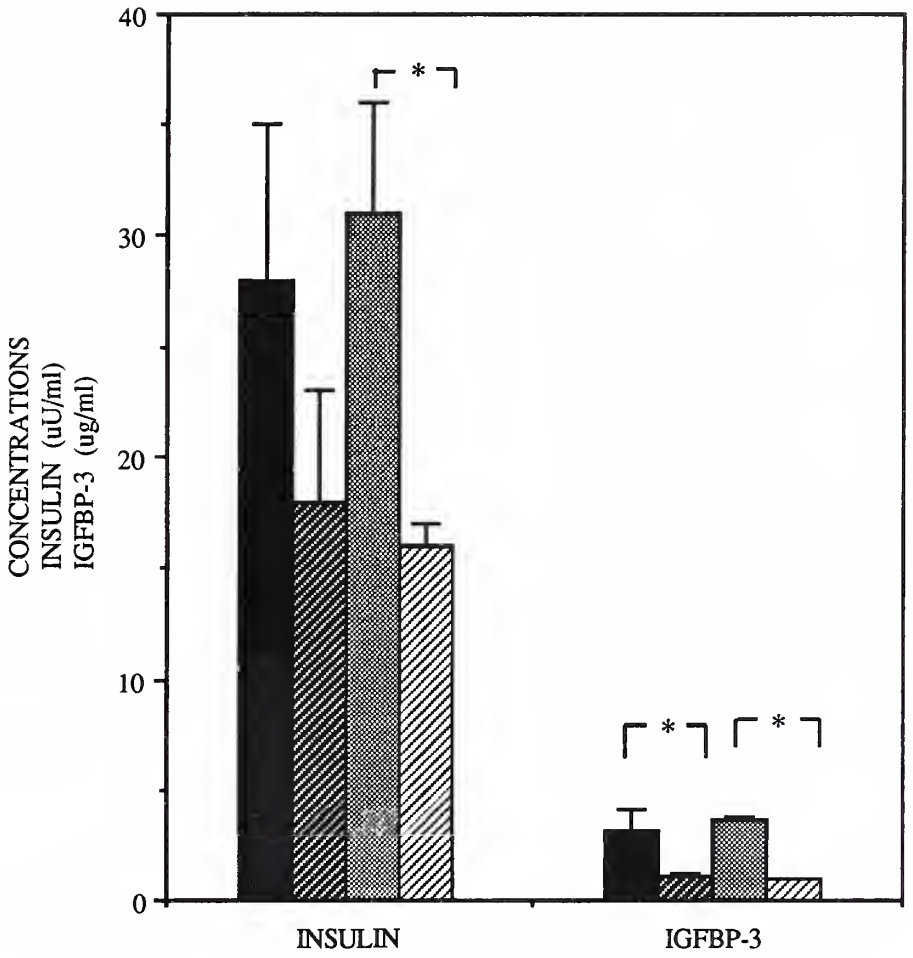

FIGURE 11: Comparison of Maternal and Neonatal Insulin and IGFBP-3 Levels within LGA and AGA Neonates

LGA - MATERNAL (n = 9) LGA - NEONATAL (n = 8)

AGA - MATERNAL (n = 18) AGA - NEONATAL (n = 18)

* $p < 0.001$

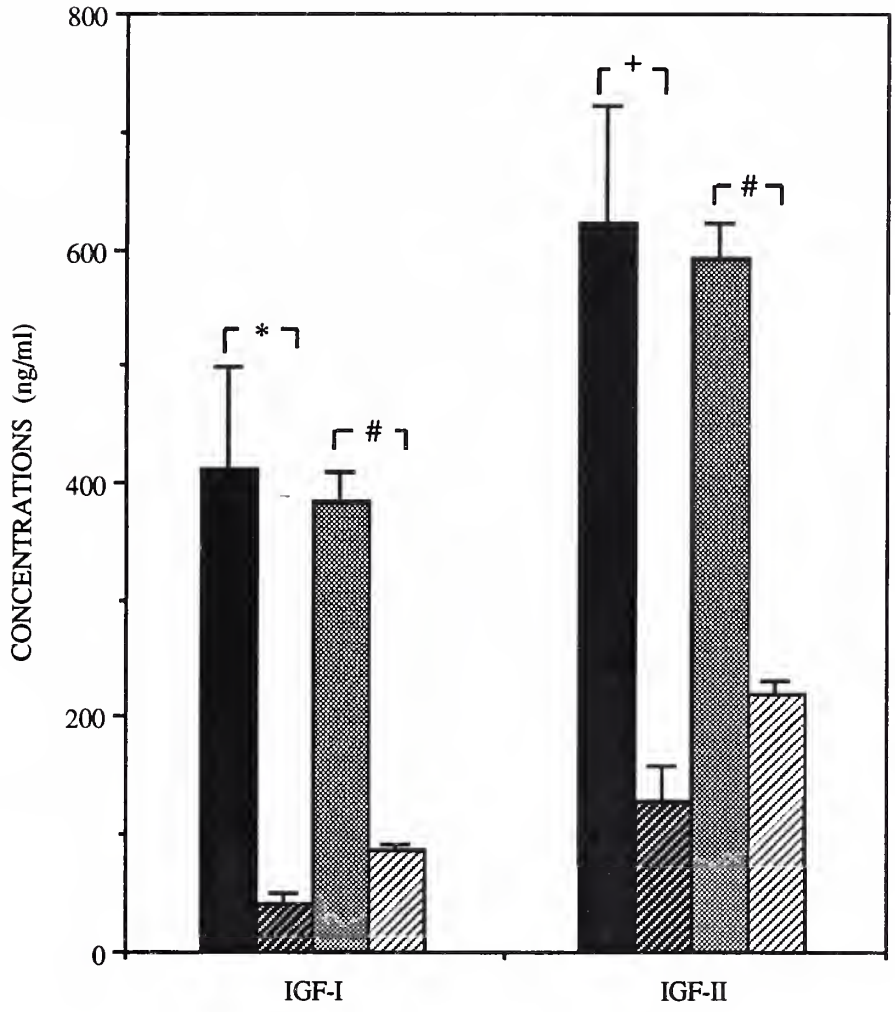

FIGURE 12: Comparison of Maternal and Neonatal IGF-I and IGF-II Levels within Small and Large Neonates

■ SMALL-MATERNAL (n = 4) ▨ SMALL-NEONATAL (n = 4)

▩ LARGE-MATERNAL (n = 23) ▨ LARGE-NEONATAL (n = 22)

* $p < 0.01$; + $p < 0.005$; # $p < 0.001$

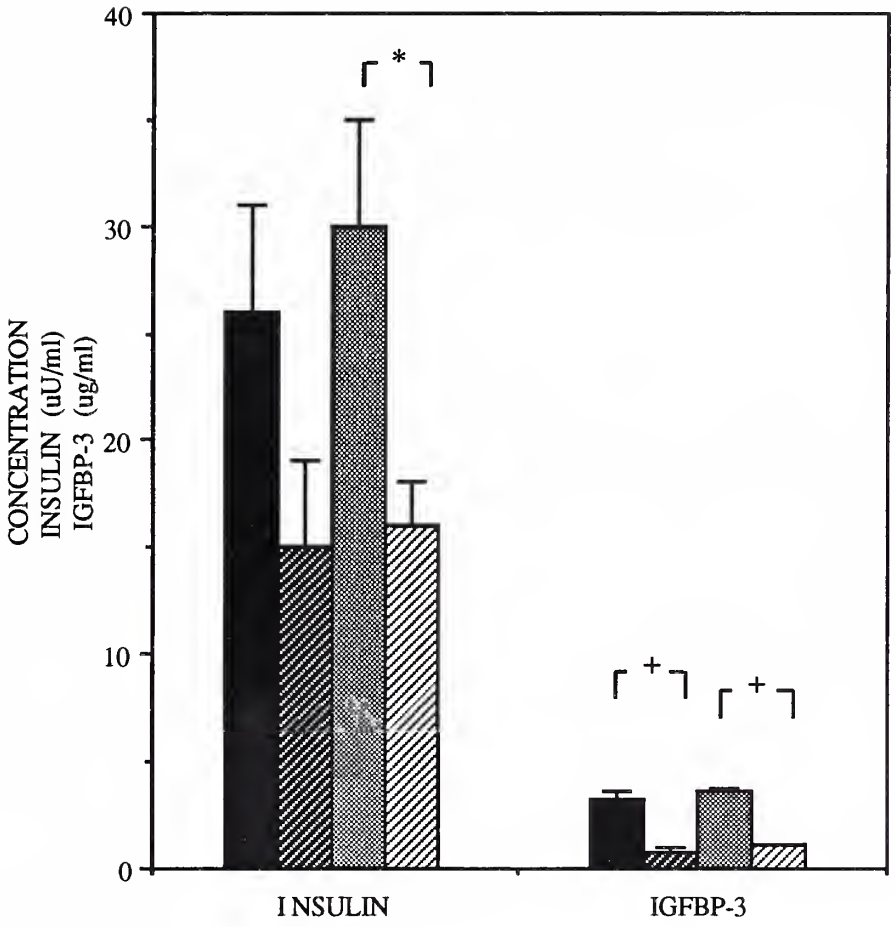

FIGURE 13: Comparison of Maternal and Neonatal Insulin and IGFBP-3 Levels within Small and Large Neonates

SMALL-MATERNAL (n =4) SMALL-NEONATAL (n = 4)

LARGE-MATERNAL (n =23) LARGE-NEONATAL (IGFBP-3; n=23 / INSULIN; n=22)

(1 data sample for insulin in the large neonatal group was not available due to technical difficulty with the assay and subsequently insufficient serum.)

* $p < 0.01$; + $p < 0.001$

	IGF-I (ng/ml)	IGF-II (ng/ml)	IGFBP-3 (μg/ml)
Maternal (n=7)	343 ± 55	510 ± 45	3.90 ± 0.20
Neonatal (n=7)	105 ± 6*	215 ± 27*	1.10 ± 0.08*
Amniotic Fluid (n=7)	124 ± 41*	299 ± 81*	1.80 ± 0.30*

TABLE 6: Maternal, Neonatal, and Amniotic Fluid Levels of IGF-I, IGF-II, and IGFBP-3 in Term AGA Infants
Mean ± SEM; * $p < 0.01$ vs. Maternal Level

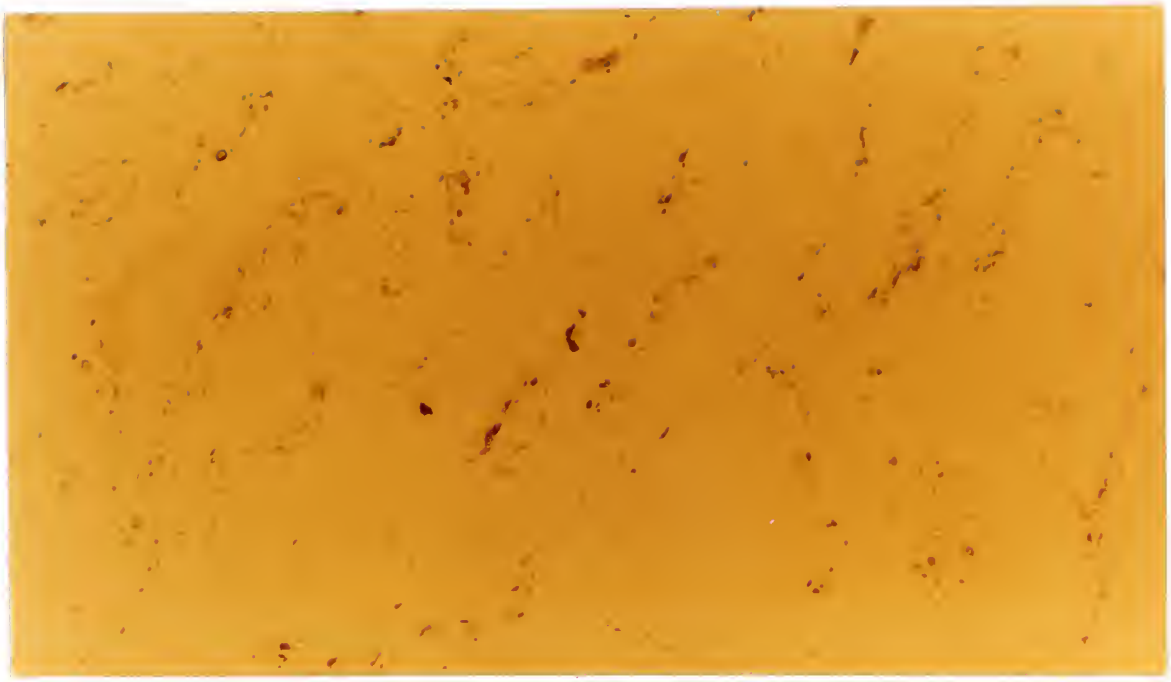

FIGURE 14: Negative Control of Insulin Receptors Assay. Primary Antibody was Omitted. Term Chorion/Decidua Tissue. 40x Magnification.

FIGURE 15: Negative Control of Insulin Receptors Assay. Secondary Antibody was Omitted. Term Placenta Tissue. 40x Magnification.

FIGURE 16: Negative Control of Insulin Receptors Assay. Streptavidin-Horseradish Peroxidase was omitted. Term Amnion Tissue. 40x Magnification.

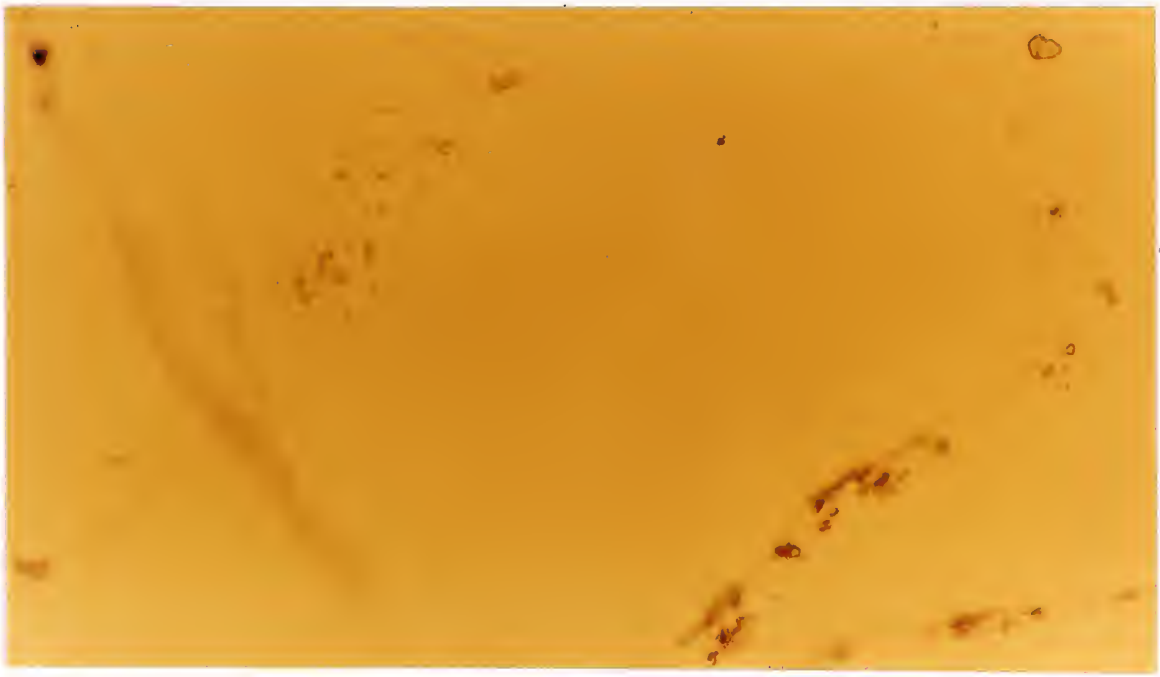

FIGURE 17: Negative Control of Insulin Receptors Assay. DABA was Omitted. Term Placenta Tissue. 40x Magnification.

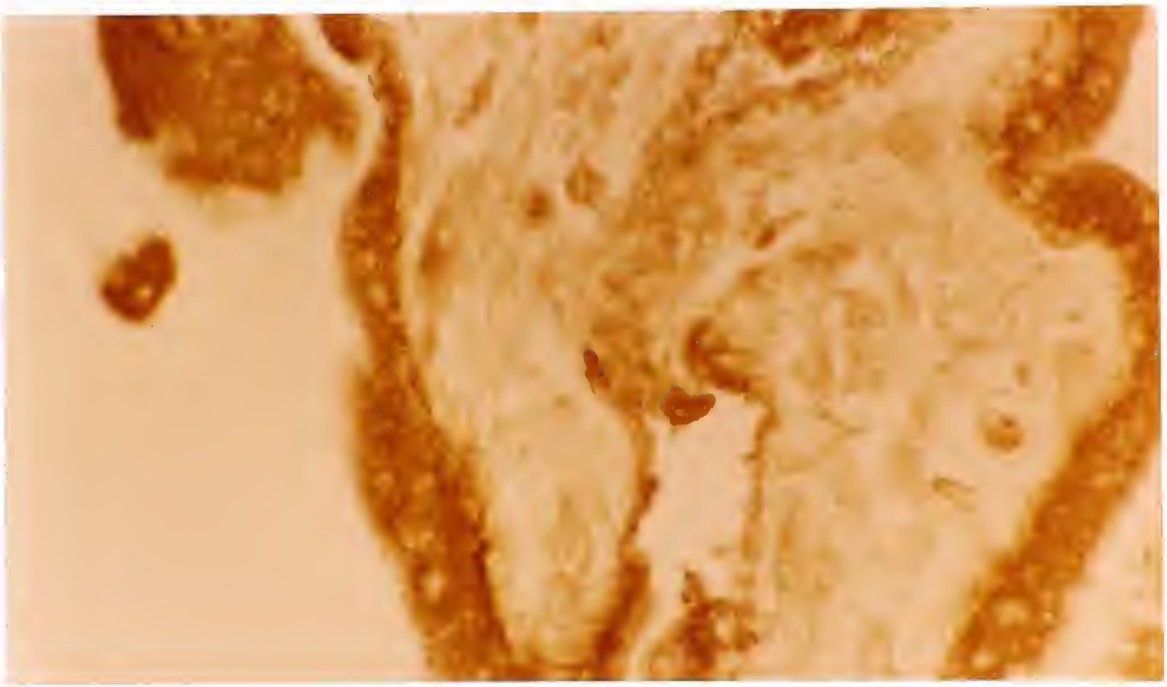

FIGURE 18: First Trimester Placenta. Strongly Positive Stained Insulin Receptors. 7/7 Samples. 20x Magnification.

FIGURE 19: Term Amnion Tissue. Strongly Positive Stained Insulin Receptors. 7/7 Samples. 40x Magnification.

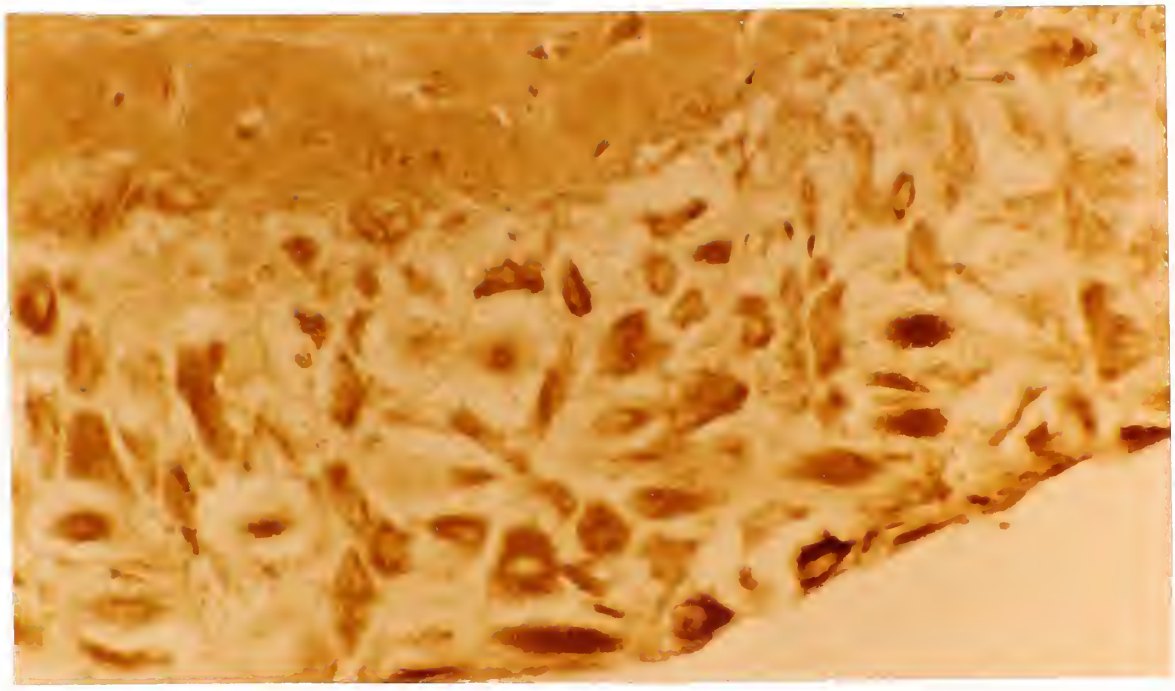

FIGURE 20: Term Chorion/Decidua Tissue. Strongly Positive Stained Insulin Receptors. 7/7 Samples. 40x Magnification.

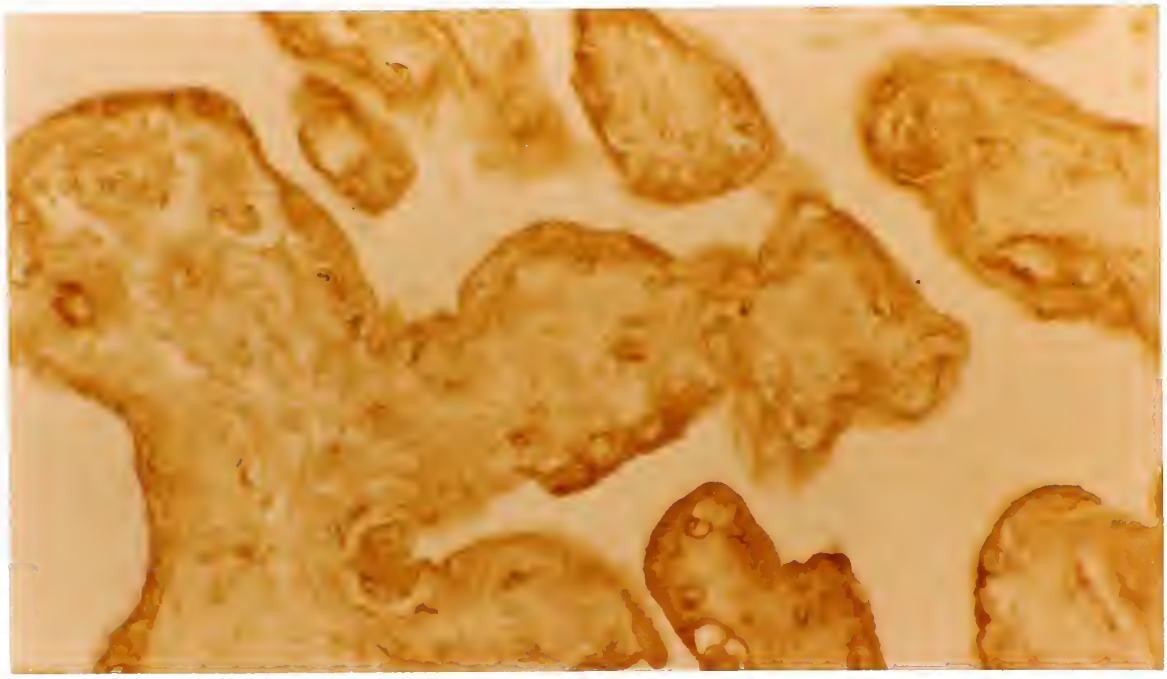

FIGURE 21: Term Placental Tissue. Moderately Positive Stained Insulin Receptors. 7/7 Samples. 40x Magnification.

FIGURE 22: Negative Control of Type 1 IGF Receptors Assay. Primary Antibody was Omitted. Term Chorion/Decidua Tissue. 40x Magnification.

FIGURE 23: Negative Control of Type 1 IGF Receptors Assay. Secondary Antibody was Omitted. Term Placenta Tissue. 40x Magnification.

FIGURE 24: Negative Control of Type 1 IGF Receptors Assay. Streptavidin-Horseradish Peroxidase was Omitted. Term Placenta Tissue. 40x Magnification.

FIGURE 25: Negative Control of Type 1 IGF Receptors Assay. DABA was Omitted. Term Placenta Tissue. 40x Magnification.

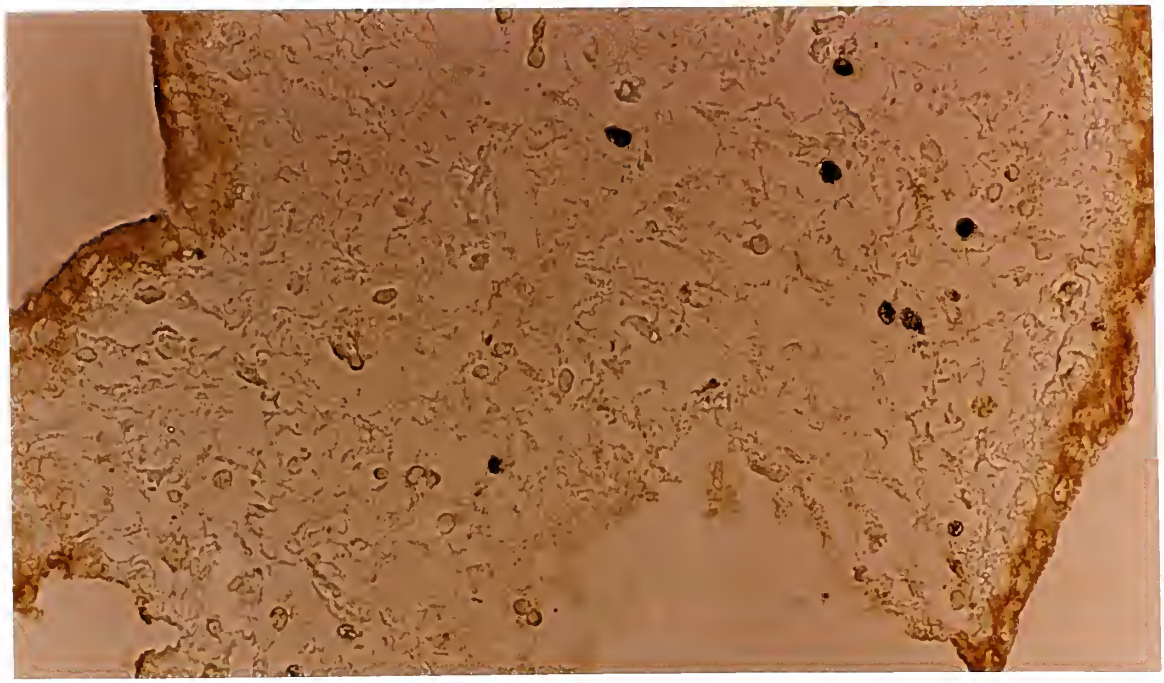

FIGURE 26: First Trimester Placenta. Strongly Positive Stained Type 1 IGF Receptors. 7/7 Samples. 40x Magnification.

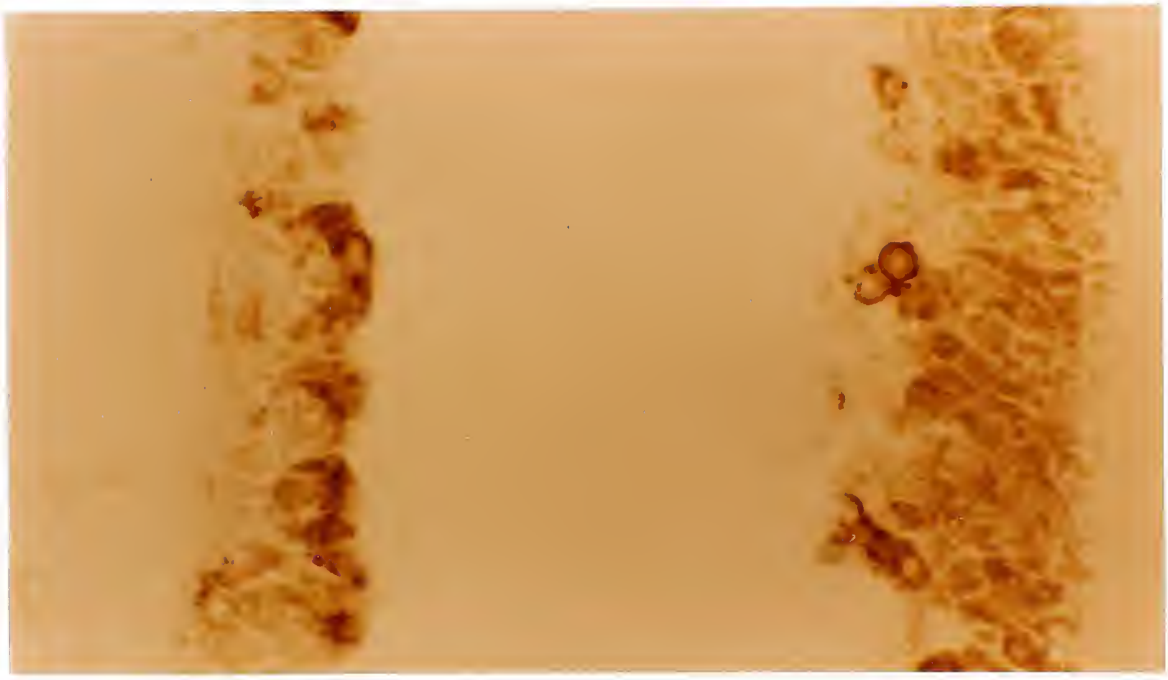

FIGURE 27: Term Amnion Tissue. Moderately Positive Stained Type 1 IGF Receptors. 7/7 Samples. 40x Magnification.

FIGURE 28: Term Chorion/Decidua Tissue. Trace Positive Stained Type 1 IGF Receptors. 4/7 Samples. 40x Magnification.

FIGURE 29: Term Placental Tissue. Slightly Positive Stained Type 1 IGF Receptors. 7/7 Samples. 40x Magnification.

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