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Expression of insulin-like growth factor II in human placentas from normal and diabetic pregnancies

(fetal growth/placental growth)

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ABSTRACT **The growth and development of the placenta is critical to fetal growth and development; however, little is known regarding the mechanisms controlling placental growth and development. Human placental membranes are known to possess receptors for insulin-like growth factors I and II (IGF-I and IGF-II) from early gestation, and increasing evidence supports a major role for IGF-I and/or IGF-II in fetal growth and development. Therefore, the IGFs may also play a significant role in regulating placental growth and development. We report here that an adult human liver IGF-II cDNA hybridizes to poly(A)$^+$ RNAs of human placentas from different gestational ages. There are four placental poly(A)$^+$ RNA species that hybridize to IGF-II cDNA, the major one of which is about 6000 bases. The sizes of the hybridized transcripts are the same for placentas of different gestational ages. Furthermore, the IGF-II sequences expressed in the human placenta were quantitated by dot blot hybridization. The second trimester placenta expresses more IGF-II mRNA sequences than placenta of first trimester and term. Interestingly, the term placentas from diabetic pregnancies also express more of these sequences than those from normal pregnancies. These results suggest that there are developmental changes in the expression of the IGF-II gene in the placenta and that IGF-II may promote placental growth by way of an autocrine and/or paracrine mechanism. Moreover, fetuses developing in diabetic pregnancies receive a large influx of glucose, which in turn may stimulate the expression of IGF-II sequences in placenta, resulting in higher utilization of glucose and overgrowth of placenta. This may explain the macrosomia and high incidence of malformations and stillbirths known to result from pregnancies in diabetics.**

The mechanism controlling human fetal growth is complex and little understood. It involves the mother and the placenta as well as the fetus. In the first 10 wk of embryonic and fetal life, primary and secondary embryonic induction and organogenesis are taking place (1); between the 14th and 25th wk of gestation, cell proliferation occurs rapidly. Thereafter, the rate of cell formation begins to slow down, and during the last 10 wk, there is rapid growth in terms of cell size (2). The growth of the placenta is related to fetal growth and follows a similar pattern.

Glucose is used as a major source of metabolic energy in the human fetus (3, 4). Insulin, a regulator of glucose metabolism, is an essential growth factor for fast-growing mammalian tissues, including human embryonic tissues (3, 4). However, the fetal pancreas begins to secrete insulin only after about 20 wk of gestation (5), and insulin secretion by the fetal pancreas exhibits only limited response to acute changes in the cord blood glucose level (6–8). In addition, maternal insulin does not cross the blood–placental barrier (9, 10). Therefore, insulin is unlikely to be the major growth factor necessary for embryonic and early fetal development.

Insulin-like growth factors I and II (IGF-I and IGF-II) are peptide mitogens that share structure homology with insulin and exhibit a similar spectrum of biological activities (11–14)—i.e., acute anabolic activity and long-term growth-promoting effects. They are thought to be critical in postnatal growth (15, 16). Studies to date have shown that IGF receptors are widespread in many fetal tissues (17, 18), and a variety of fetal tissues respond to (19, 20) and produce IGF (21–23). In addition, the concentrations of IGF in fetal and cord blood are correlated with fetal birth size (24). These observations suggest that the IGF may play an important role in fetal growth and development. Since maternal IGF does not cross the blood–placental barrier (25), the fetal IGF must be synthesized by the feto-placental unit.

In recent studies on the synthesis and secretion of placental proteins in normal and diabetic pregnancies, we have observed the expression of insulin-related sequences and IGF-I sequences, but no expression of insulin, in the placenta (26, §). By using human adult liver IGF-II cDNA as probe, we report here that the IGF-II gene is expressed in the human placenta throughout the gestational period in normal and diabetic pregnancies.

METHODS

Preparation of Total RNA and Poly(A)$^+$ RNA from Placenta. Placentas were obtained within 20 min of delivery at the MacDonald Hospital for Women, University Hospitals of Cleveland, from normal pregnant women at term, diabetic pregnant women at term or near term, and preterm therapeutic abortions. The term and near-term placentas were obtained primarily from cesarean sections. Diabetics were classified according to White (27). Procurement of tissue was approved by the committee for the protection of human subjects of Case Western Reserve University and University Hospitals of Cleveland. Placentas were frozen in liquid nitrogen and stored at −80°C or processed immediately. First trimester placentas (8–12 wk of gestation) were pooled. Total RNA was isolated by using the guanidine thiocyanate (Fluka, purum) procedure of Chirgwin *et al.* (28), with the exception that the tissue/guanidine thiocyanate solution ratio was 1:4 (g:ml). Poly(A)$^+$ RNA was prepared from total RNA by passing twice on oligo(dT)-cellulose chromatography columns (type 3, Collaborative Research, Waltham, MA) (29).

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Abbreviations: IGF, insulin-like growth factor; hPL, human placental lactogen.

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RNA Transfer Blot Analysis. The pIGF-II isolated from an adult human liver cDNA library (30) was restriction digested with *Pst* I to obtain the IGF-II cDNA insert, and the G/C tails of the cDNA insert were removed by nuclease BAL-31. The final IGF-II cDNA insert was 660 base pairs and contained the coding region of the IGF-II precursor flanked by the 3'-nontranslated region of 190 base pairs. It was nick-translated with [α-^{32}P]dCTP in the absence of DNase I.

The placental poly(A)$^+$ RNAs from different gestational ages were electrophoresed in a 1% agarose minigel (15-ml gel volume) using a formaldehyde denaturing procedure (31), transferred to a nitrocellulose membrane in 3 M NaCl/0.3 M sodium citrate, pH 7.0, and baked in a vacuum oven for 2 hr at 80°C. The *Hin*dIII restriction fragments of λ DNA were electrophoresed in an adjacent lane and stained with ethidium bromide to serve as size markers. The hybridization was carried out with ^{32}P-labeled IGF-II cDNA insert (2–5 × 10^7 cpm/μg, 1 × 10^6 cpm/ml) at 42°C in a solution containing 50% (vol/vol) formamide, 0.9 M NaCl/90 mM sodium citrate, 0.1 M sodium phosphate (pH 7.2), 1× Denhardt's solution (0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 20 μg of sonicated and denatured *Escherichia coli* DNA per ml, 10% dextran sulfate, and 0.1% $NaDodSO_4$. The blot was washed in 0.15 M NaCl/15 mM sodium citrate and 0.1% $NaDodSO_4$ at 65°C and autoradiographed.

RNA Dot Blot Analysis. The placental RNAs were denatured at 60°C for 15 min in a 100-μl solution containing 7.4% (wt/wt) formaldehyde and 0.9 M NaCl/90 mM sodium citrate; they were then chilled on ice, and 300 μl of 1.5 M NaCl/0.15 M sodium citrate was added. The RNA samples were applied to a nitrocellulose membrane using a Minifold apparatus (Schleicher & Schuell) under vacuum. After sample application, the blot was baked for 2 hr at 80°C in a vacuum oven. In the control experiments, the RNA samples were heated in 0.05 M NaOH (pH 12.5) for 1 hr at 65°C and then neutralized with 0.2 M HCl before the dot blotting procedure. The hybridization conditions were the same as for the RNA transfer blot analysis.

RESULTS

Total and poly(A)$^+$ RNAs were extracted from placentas at various stages of normal gestation as well as from term placentas of diabetic mothers. To determine whether placenta expresses IGF-II sequences, placental poly(A)$^+$ RNAs were separated by electrophoresis on agarose gels, transferred to a nitrocellulose membrane, and hybridized to a ^{32}P-labeled adult human liver IGF-II cDNA insert. Under high stringency conditions (90% + homology), there are four placental poly(A)$^+$ RNA defined species (6000, 4900, 3200, and 2200 bases) that hybridize to the IGF-II cDNA insert, the major of which is 6000 bases, indicating that the IGF-II mRNA is expressed in the placenta (Fig. 1). The results also revealed that there is no size difference in IGF-II mRNA species expressed in placentas of different gestational ages and in placentas of normal and diabetic pregnancies. However, there is a quantitative difference in the expression of IGF-II sequences in the placenta.

Since the transfer of RNA from agarose gel to nitrocellulose membrane is always incomplete, dot blot analysis of placental poly(A)$^+$ RNA (Fig. 2) and total RNA (Fig. 3) was carried out to quantify more carefully and accurately the IGF-II mRNA expressed in the placenta. The RNA was applied in 1:2 serial dilutions to the nitrocellulose membrane and hybridized with ^{32}P-labeled IGF-II cDNA insert. In the control experiments, all RNA samples were treated with NaOH prior to dot blot analysis to account for the possible contamination of chromosomal DNA. In the poly(A)$^+$ RNA dot blot, no DNA contamination was found. However, in the total RNA dot blot, there was a small degree of DNA contamination in a few samples (data not shown), and the overall background was higher than in the poly(A)$^+$ RNA dot blot. This may have been due to hybridization with rRNA. For the quantification, the autoradiograms of the dot blots were scanned densitometrically at 540 nm and the areas were integrated. The dots were also cut from the membrane and assayed in a β scintillation counter. Both quantitative meth-

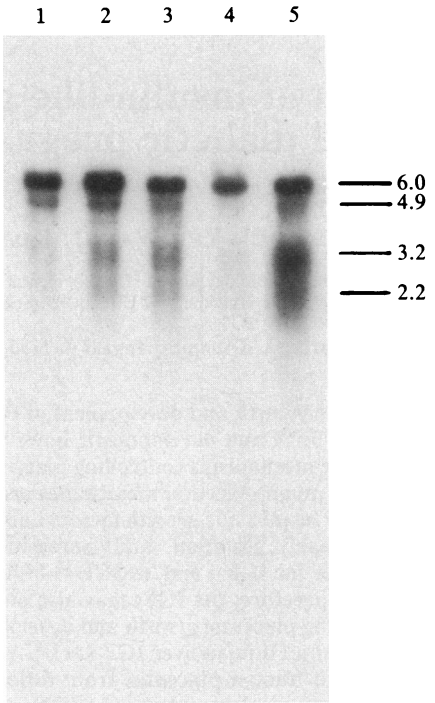

FIG. 1. RNA transfer blot analysis of placental poly(A)$^+$ RNA from different gestational ages hybridized to human adult liver IGF-II cDNA. The gestational ages of placental poly(A)$^+$ RNA are 11–12 wk (lane 1), 29 wk (lane 2), normal term (lane 3), term from gestational diabetic pregnancy (lane 4), and term from overt diabetic (class B) pregnancy (lane 5). Each lane contains 2.5 μg of poly(A)$^+$ RNA except lane 2, which contains 3 μg of poly(A)$^+$ RNA. The sizes of hybridized transcripts are indicated in kilobases. The *Hin*dIII fragments of λ DNA were used as size standards.

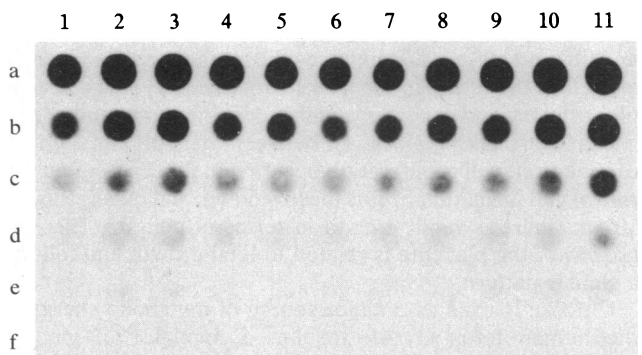

FIG. 2. Dot blot analysis of placental poly(A)$^+$ RNA from different gestational ages hybridized to human adult liver IGF-II cDNA. The gestational ages of placental poly(A)$^+$ RNA are 11–12 wk (columns 1 and 2), 29 wk (column 3), normal term (columns 4–7), term from gestational diabetic pregnancy (columns 8–10), and term from overt diabetic (class B) pregnancy (column 11). The poly(A)$^+$ RNA samples were applied in 1:2 serial dilutions from rows a to f using the following amounts: 2 μg, 1 μg, 0.5 μg, 0.25 μg, 0.125 μg, and 0.0625 μg, respectively. All samples contained 10 μg of RNA with yeast tRNA as carrier. Ten micrograms of yeast tRNA did not give any background in dot blot hybridization.

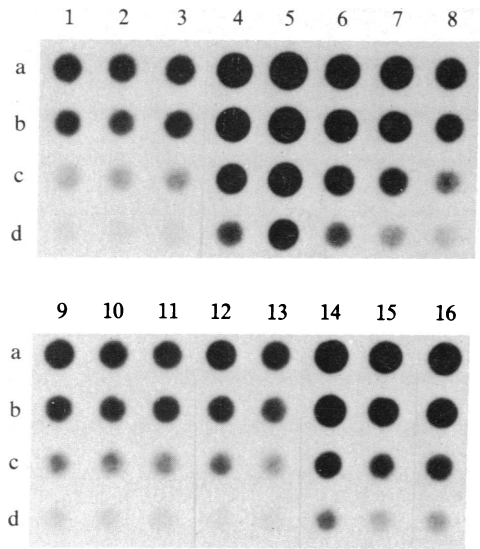

FIG. 3. Dot blot analysis of placental total RNA from different gestational ages hybridized to human adult liver IGF-II cDNA. The gestational ages of placental total RNA are 8–9 wk (column 1), 9–10 wk (column 2), 11–12 wk (column 3), 19–20 wk (column 4), 21 wk (column 5), 24 wk (column 6), 29 wk (column 7), 31 wk (column 8), normal term (columns 9–11), term from gestational diabetic pregnancy (columns 12–15), and term from overt diabetic (class B) pregnancy (column 16). The total RNA samples were applied in 1:2 serial dilutions from rows a to d using the following amount: 40 μg, 20 μg, 10 μg, and 5 μg, respectively.

ods gave similar results. Table 1 shows the relative abundance of IGF-II mRNA in the placenta after correction for nonspecific binding and DNA contamination. The results of the dot blot analysis for total RNA and poly(A)$^+$ RNA are consistent, indicating that these are not an artifact resulting from the contamination of chromosomal DNA and/or rRNA. According to the dot blot analysis of poly(A)$^+$ RNA and total RNA, the second trimester placentas (19–29 wk of gestation) express more IGF-II mRNA than those of first trimester (8–12 wk of gestation) and normal term, and the peak of the IGF-II mRNA expression in the placenta is around 21 wk of gestation. The first trimester placenta (villus trophoblast) has a similar amount or slightly less IGF-II mRNA than term placenta. These results suggest that there are developmental changes in the expression of the IGF-II gene in the placenta. Furthermore, the term placentas from class A diabetics (gestational diabetes, a form of diabetes which arises from and is confined to pregnancy) and class B overt diabetics contain more IGF-II mRNA than those of normal pregnancies. In addition, although only one placenta from a class B diabetic mother was analyzed, it appeared to contain more IGF-II than those of gestational diabetic mothers. However, additional data are required before conclusions can be made. There are variations in the data obtained from gestational diabetics. Some placentas of gestational diabetic pregnancies contain about the same amount of IGF-II mRNA as those of normal pregnancies (Fig. 3, columns 12 and 13), and some contain much more IGF-II mRNA than those of normal pregnancies (Fig. 3, columns 14 and 15). This may be due to the different degree of severity in the diabetic condition, since gestational diabetes is defined by an abnormal glucose tolerance test during pregnancy. However, it is known that the amount of total RNA in placentas of gestational and overt diabetic pregnancies is significantly elevated over normal term placentas per gram of tissue (32). Therefore, the level of IGF-II mRNA in placentas of diabetic pregnancies, which also always have larger placentas (33), is substantially higher than those of normal pregnancies.

Table 1. Relative abundance of IGF-II mRNA in placentas of different gestational ages

Placental gestational age, wk	Relative abundance	
	Poly(A)$^+$ RNA	Total RNA
8–9	—	0.74 (1)
9–10	—	0.78 (1)
11–12	1.04 (2)	0.87 (1)
19–20	—	2.11 (1)
21	—	3.05 (1)
24	—	1.89 (1)
29	1.5 (1)	1.44 (1)
31	—	0.94 (1)
Term		
Normal	1.00 (4)	1.00 (3)
Gestational diabetics	1.20 (3)	1.08 (4)
Overt diabetics	1.87 (1)	1.42 (1)

Relative levels of IGF-II mRNA were determined by integrating the areas of densitometer scans of the dot blot analysis autoradiograms. The values are normalized to those of normal term placenta. Values in parentheses indicate the number of RNA preparations used in this analysis.

DISCUSSION

Many hormones and regulatory factors are known to be synthesized by the human placenta. It is not surprising, therefore, that the placenta is capable of synthesizing IGF-II, a growth factor thought to be important in fetal growth (34). The large size of the major IGF-II transcript (6000 bases) present in placenta is surprising because prepro-IGF-II is encoded by only 540 bases (35). However, this is not unusual since the IGF-II mRNAs expressed in adult liver and kidney also have large molecular masses and are different in size (36). This may indicate tissue-specific processing of IGF-II mRNA. The three less abundant IGF-II mRNA species expressed in placenta may result from alternative RNA processing from a single primary transcript, since only one IGF-II gene is present in the human genome (36), or they may represent other IGF-II-related mRNAs expressed in the placenta.

The IGF-II synthesized in placenta may play several roles. There is evidence that IGF can act through an autocrine and/or paracrine mechanism—that is, on its cells of origin or on cells in close proximity (37). The receptors specific for IGF-II have been found on placental membranes from early gestation (38). Deal *et al.* (39) have demonstrated that the specific binding of insulin-like activity on placental membrane is higher during the midterm (11.5–19 wk) of gestation than at term, which correlates with our finding that placentas of second trimester express more IGF-II mRNA than term placentas. Therefore, it is reasonable to hypothesize that the IGF-II produced by the placenta acts locally by way of an autocrine and/or paracrine mechanism to promote placental growth. Furthermore, the placental IGF-II may play a role in fetal growth and development. Some evidence in experimental animals points to a role for IGF-II as a fetal growth factor that is replaced by IGF-I around the time of birth (22). Sara *et al.* (40) suggested that IGF regulates the early proliferative phase of fetal growth, whereas insulin may influence later hypertrophic growth. In our experiments, the placentas of diabetic pregnancies contain more IGF-II mRNA than those of normal pregnancies. It is known that glucose is freely available to the fetus from the maternal circulation and is supplied at higher rates to the fetus in a diabetic pregnancy (41). Therefore, the high concentration of glucose in the fetuses of diabetic pregnancies may increase the level of the placental IGF-II mRNA, which may in turn stimulate the glucose utilization and/or mitosis of placental cells, resulting in overgrowth of placenta. This may have some effect on fetal

growth since fetal growth is dependent upon placental growth. Alternately, the placental IGF-II may stimulate the glucose utilization and cell proliferation in the fetus itself. Further evidence for this hypothesis comes from the finding of Widdowson *et al.* (2) that in fetal organs all proliferation occurred rapidly between 14 to 25 wk of gestation and from the report of Hendricks (42) that the percentage of fetal growth increments are higher during second trimester and decline rapidly until term.

The mechanism for regulation of IGF-II synthesis in the placenta is unclear. However, there is persuasive evidence that during pregnancy, human placental lactogen (hPL) substitutes for growth hormone in the regulation of IGF synthesis and secretion (34). In addition, the level of the multiplication-stimulating activity (rat IGF-II) in fetal serum increased when malnourished mother rats were infused with hPL (43). Adams *et al.* (22) demonstrated that ovine PL stimulates IGF-II synthesis in rat fetal fibroblasts. Furthermore, in a diabetic pregnancy, the placenta expresses more hPL mRNA (32), which may bring about higher levels of IGF-II mRNA. There is a close relationship between maternal hPL levels and placental weight from the 12th wk of gestation (44). From these observations, it is likely that hPL may at least partially regulate the synthesis of IGF-II in the placenta, which in turn may regulate placental and/or fetal growth.

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