At late fetal age (21.5 days postcoitum [dpc]), GK rats present a severely reduced β-cell mass compared with Wistar rats. This anomaly largely antedates the onset of hyperglycemia in GK rats. Thus, the β-cell mass deficit could represent the primary defect leading to type 2 diabetes in the adult. The aim of this work was to investigate, in GK fetuses at the end of fetal age (21.5 dpc), whether impaired availability of growth factors such as insulin, growth hormone, and IGFs and their IGF binding proteins (IGFBPs) could be instrumental in this anomaly. Although it confirms that GK fetuses are hypoinsulinemic despite enhanced plasma glucose level due to maternal hyperglycemia, the present study shows for the first time that IGF-2 expression in the liver and pancreas and IGF-2 serum levels are decreased in GK fetuses. Serum level as well as liver and pancreatic mRNA expression of IGFBP-2 were found to be normal in GK fetuses, whereas serum level and liver mRNA expression of IGFBP-1 were increased. Finally, we found that the maximal β-cell mitogenic response to IGFs in vitro is kept intact, therefore suggesting that the direct biological action of IGFs on fetal GK β-cells is not grossly impaired. In conclusion, in GK fetuses at 21.5 dpc, the defective IGF-2 production appears to be an early landmark in the pathological sequence leading to retardation of β-cell growth in the fetal GK rat. 

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**At late fetal age (21.5 days postcoitum [dpc]), GK rats present a drastically reduced β-cell mass compared with Wistar rats (1,2). This anomaly largely antedates the onset of hyperglycemia in the GK rats. Thus, the β-cell mass deficit could represent the primary defect leading to type 2 diabetes in the adult. Recent data (3) suggested that the impaired development of the GK pancreas probably results from insufficiency of an extrapancreatic factor(s) necessary to the growth and survival of fetal pancreatic cells. IGF-1 and -2 are single-chain polypeptides that have structural homology to pro-insulin, although they are encoded by individual genes. IGF-1 and -2 are essential cell growth regulators, as demonstrated by null mutation experiments (4). They are involved in cell proliferation and differentiation of multiple tissues and organs (4). IGFs are synthesized primarily by the liver, but they are also produced locally by many tissues, where they act in an autocrine or paracrine manner. In the rat, IGF-2 is expressed at high levels during embryonic development, but its expression progressively extinguishes in most tissues after birth, except in brain (5). The IGF-1 gene is also expressed in a variety of fetal rat tissues, although at lower levels than the IGF-2 gene. IGF-1 and -2 mRNA transcripts are present in virtually all fetal tissues, and direct evidence of a role for IGF-2 in fetal growth has been demonstrated (5). They are present in serum and other extracellular fluids associated with highly specific binding proteins (IGF binding proteins [IGFBPs]). In the fetus, IGFs are predominantly complexed with IGFBP-1 and -2 (6), and the liver is the predominant production site for these IGFBPs (7). During the fetal period, insulin also regulates growth, and IGF regulation is growth hormone (GH)-independent (5). Because there is considerable evidence that endocrine factors such as insulin, GH, and IGFs contribute to β-cell growth as well as maturation and function throughout life (5) and that IGFs actions can be modulated by locally produced IGFBP, the aim of this work was to investigate in GK fetuses at the end of fetal life (21.5 dpc): I) the circulating levels of insulin, GH, IGFs, and IGFBP-1 and -2; 2) the expression of IGFs and IGFBP-1 and -2 mRNAs in liver and pancreas; and 3) the in vitro mitogenic effect of IGFs in isolated fetal islets.**
standard acid gel filtration. This method has proved to be the most reliable one for use with rat serum in developing stages (9,10).

The radioimmunoassay for IGF-I and rat liver membrane receptor assay for IGF-2 were carried out as previously described (9,10). The coefficients of variation within and between assay were 8.9% and 12.4%, respectively. Recombinant human IGF-I and -II (Boehringer Mannheim, Mannheim, Germany) were used for iodination.

**Western immunoblotting and determination of serum IGFBP-1 and -2.** Western immunoblots for enhanced chemiluminescence were performed in polyvinylidene fluoride (PVDF) immobilon-P membranes (Millipore, Billerica, MA). PVDF membranes were blocked with 5% (wt/vol) nonfat dry milk for 60 min in Tris-buffered saline (TBS; 0.01 mol/l Tris and NaCl 0.15 mol/l, pH 8) with 0.05% Tween-20. Membranes were then incubated with a 1:1,000 dilution (as suggested by the manufacturer) of affinity-purified goat polyclonal anti-rat IGFBP-1 or –rat IGFBP-2 (Santa Cruz Biotechnology, Madrid, Spain) in the same buffer (TBS-Tween plus 5% nonfat dry milk) at 4°C overnight, after which the membrane was washed three times for 10 min in TBS-Tween. After a 1-h incubation at room temperature with a 1:1,000 dilution of anti-goat IgG–horseradish peroxidase in TBS-Tween plus 5% nonfat dry milk, the membrane was washed three times with TBS-Tween and finally once with TBS alone. Antigen-antibody complexes were detected after an enhanced chemiluminescence (hyperfilm ECL, Amersham, Amersham, Madrid).

**Preparation of total RNA.** Total RNA was prepared by homogenization of fetal pancreas and livers in guanidinium thiocyanate as originally described (11). RNA concentration was determined by absorbance at 260 nm. Samples were electrophoresed through 1% agarose and 2 mol/l formaldehyde gels and then stained with ethidium bromide to render the 28S and 18S ribosomal RNA visible and thereby confirm the integrity of the RNA and normalize the quantity of RNA in the different lanes. A pT7 RNA 18S anti-sense (Ambion, Austin, TX) was used for lane loading control.

**Riboprobes.** Rat IGF-I and -2 and IGFBP-1 and -2 cDNAs were kindly provided by Drs. C.T. Roberts Jr. and D. LeRoith (National Institutes of Health, Bethesda, MD). Rat IGF-I cDNA ligated into a pGEM-3 plasmid (Promega Biotech, Madison, WI) was linearized with HindIII, and an antisense riboprobe was produced by T7 RNA polymerase. The size of the protected fragment represented in the figures (IGF-Bb) was 385 bp. Rat IGF-II cDNA ligated into a pGEM-3 plasmid was linearized with HindIII and incubated with T7 RNA polymerase to generate a riboprobe that recognized a fragment of 700 bp. Rat IGF-BP-1 cDNA, ligated into a pGEM-5 plasmid, was linearized with HindIII and incubated with T7 RNA polymerase to generate an anti-sense riboprobe that recognizes two fragments of 300 and 700 bases. Rat IGBP5 cDNA, ligated into a pGEM-4Z (Promega Biotech, Madison, WI), was linearized with HindIII and incubated with SP6 RNA polymerase to generate a 550 base anti-sense riboprobe devoid of pGEM-4Z complementary sequences. pT7 RNA 18S was incubated with T7 RNA polymerase to produce a 100 nucleotide runoff transcript, 90 nucleotides of which are complementary to human 18S ribosomal RNA. 5′-P-UTP was purchased from ICN (Nuclear Iberica, Madrid). The Riboprobe Gemini II Core System (Promega) was used for the generation of RNA probes.

**Solution hybridization/RNAse protection assay.** Solution hybridization/RNAse protection assays were performed as previously described (10,12). Autoradiography was performed at –70°C against a Hyperfilm MP film between intensifying screens. Bands representing protected probe fragments were quantified using a Molecular Dynamics scanning densitometer and accompanying software. RNAse A and T1 were purchased from Boehringer Mannheim.

**Fetal rat islet preparation and islet culture with IGFs.** Fetal islets from GK and Wistar rats were prepared according to Hellerström et al. (13) as previously described (8). At the end of the 6-day culture period, 40 fetal islets in each group were collected under a stereomicroscope and further cultured for 2 days in RPMI 1640 medium (Bio Whittaker, Verviers, Belgium) supplemented with 2 mmol/l glutamine (Bio Whittaker), 1% heat-inactivated fetal bovine serum (Bio Whittaker), and 100 ng/ml IGF-1 (R&D Systems, Abingdon, U.K.) or 100 ng/ml IGF-2 (R&D Systems). The culture dishes were kept at 37°C in a humidified atmosphere of 5% CO2 in air. The complete culture medium was changed the next day.

**β-Cell replication.** To measure β-cell replication in isolated fetal islets, 5′-bromo-2′-deoxyuridine (BrdU) (Amersham, Amersham, U.K.) was incorporated in newly synthesized DNA and therefore labeled replicating cells. In each group of fetal islets, 1 h before the end of islet cultures, BrdU was added at 100 μmol/l final concentration. Thereafter, islets were collected under stereomicroscope, fixed, and then processed for serial sections as previously described (8). Islets sections were double-stained with BrdU, using a cell proliferation kit (Amersham International, Amersham, U.K.), and insulin. Sections were incubated with a mouse monoclonal antibody anti-BrdU diluted in a nuclease solution (according to the kit protocol) for 1 h at room temperature and washed with Tris 0.05 mol/l, pH 7.6. Thereafter, they were incubated with an affinity-purified peroxidase anti-mouse IgG and stained with DAB using a peroxidase substrate kit 3,3′-diaminobenzidine-tetra-hydrochloride (DAB) (Dako). Sections were mounted in Eukitt (Labonord, Templemars, France). The activity of the antibody-alkaline phosphatase complex was revealed with an alkaline phosphatase substrate kit (Valbiotech, Paris, France). Sections were mounted in Eukitt (Labonord, Templemars, France). On these sections, β-cells showed red cytoxis, and BrdU-positive β-cell appeared with brown nuclei. A mean of 250 β-cells were counted per islet at a final magnification of 1000×. The proportion of BrdU-positive β-cell nuclei to total β-cell nuclei was calculated. The result represents the percentage β-cell replicative rate in a 1-h interval (BrdU labeling index of β-cells).

**Statistical analysis.** All data are presented as means ± SE. Comparison between groups was evaluated using Student’s unpaired t test. P < 0.05 was considered significant.

### RESULTS

**Biological characteristics of Wistar and GK fetuses at 21.5 dpc.** Body weight was similar in the two groups of fetuses (Table 1). GK pancreases used for RNase protection assay (determination of IGF and IGFBP mRNA expression) showed a lower (P < 0.001) weight than Wistar pancreases. By contrast, GK fetuses exhibited a higher plasma glucose concentration and a lower plasma insulin level (P < 0.001) as compared with Wistar fetuses. Plasma GH levels in GK fetuses were slightly increased (P < 0.05) as compared with Wistar levels.

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Pancreas weight (mg)</th>
<th>Plasma glucose (g/l)</th>
<th>Plasma insulin (ng/ml)</th>
<th>Plasma GH (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar</td>
<td>4.7 ± 0.1 (59)</td>
<td>24.7 ± 1.0 (14)</td>
<td>0.72 ± 0.02 (46)</td>
<td>21.8 ± 2.4 (46)</td>
<td>99.8 ± 4.6 (40)</td>
</tr>
<tr>
<td>GK</td>
<td>4.6 ± 0.1 (45)</td>
<td>19.5 ± 0.6 (15)</td>
<td>1.15 ± 0.04 (45)</td>
<td>6.3 ± 0.4 (45)</td>
<td>115.7 ± 6.5 (41)</td>
</tr>
</tbody>
</table>

Data are means ± SE. Number of determinations is shown in parentheses. Fetuses were obtained from 4–13 different litters. *P < 0.001 and †P < 0.05 as compared with Wistar fetuses.

**Statistical analysis.** All data are presented as means ± SE. Comparison between groups was evaluated using Student’s unpaired t test. P < 0.05 was considered significant.
Liver IGF and IGFBP mRNA expression in Wistar and GK fetuses. The weight of liver samples used for RNase protection assay (determination of IGF and IGFBP mRNA expression) was similar in Wistar and GK fetuses (121.7 ± 9.9 mg, n = 12, and 105.7 ± 6.7 mg, n = 14, respectively) (Fig. 2).

Densitometric measurements of protected probe fragments are expressed as percent of the corresponding Wistar values. Liver IGF-1 and -2 mRNA expression was similar in Wistar and GK fetuses (121.7 ± 9.9 mg, n = 12, and 105.7 ± 6.7 mg, n = 14, respectively) (Fig. 2).

Pancreas IGF and IGFBP mRNA expression in Wistar and GK fetuses. Denimotometric measurements of protected probe fragments are expressed as percent of the corresponding Wistar values. Pancreas IGF-1 mRNA expression in GK fetuses was similar to that in Wistar fetuses, IGFBP-1 and -2 mRNA expression was increased (P < 0.01) in GK fetuses as compared with Wistar fetuses, IGFBP-2 mRNA expression was similar in the two groups of fetuses.

In vitro mitogenic effect of IGFs in isolated fetal islets. The number of β-cells per isolated fetal islet from GK rats was significantly lower as compared with that in Wistar rats (402 ± 34 β-cell/islet, n = 43, vs. 560 ± 44 β-cell/islet, n = 50, P < 0.01) (Fig. 4). BrdU labeling index of β-cells in absence of IGF was similar in Wistar and GK-isolated fetal islets (1.12 ± 0.18%, n = 15, and 1.27 ± 0.14%, n = 14, respectively). Addition of IGF-1 or -2 to the Wistar-isolated fetal islets significantly increased (1.68 ± 0.19%, n = 14, and 1.68 ± 0.17%, n = 14, respectively, P < 0.05) the β-cell replication above the basal values (without IGF). In Wistar islets, no difference was observed between the in vitro mitogenic effect of IGF-1 or -2. Similarly, addition of IGF-1 or -2 to the GK-isolated fetal islets significantly increased (2.05 ± 0.22%, n = 19, and 2.37 ± 0.25%, n = 17, P < 0.01) the β-cell replication above the basal values (without IGF).
At late fetal age (21.5 dpc), GK rats present a severely reduced β-cell mass compared with Wistar rats (1,2). The aim of this work was to investigate whether impaired availability of growth factors such as insulin, GH, and IGFs and their IGFBPs could be instrumental in this anomaly.

Although it confirms that GK fetuses are hypoinsulinemic despite enhanced plasma glucose level due to maternal hyperglycemia, the present study shows for the first time that IGF-2 expression in the liver and pancreas and IGF-2 serum levels are decreased in GK fetuses.

IGFs are locally produced by pancreas, where they act in an autocrine or paracrine manner and are involved in the regulation of islet growth and differentiation (5). We have previously shown that the reduced β-cell mass in GK fetuses is due to an early impaired rate of β-cell neogenesis, because poor proliferation and/or survival of endocrine precursor cells leads to early defective development of the β-cell mass (3). Thus, the reduced IGF-2 serum level together with the reduced expression of IGF-2 in pancreas of GK fetuses could play a crucial role in the anomaly of β-cell mass in the GK fetuses. This is substantiated by the recent demonstration that an increased expression of IGF-2 under the control of the rat insulin promoter in β-cells of transgenic mice led to β-cell hyperplasia (14). In a rat model of increased islet number induced by high-carbohydrate feeding in the neonate, IGF-2 expression was found increased within the pancreatic ductal epithelium, and it may contribute to the associated higher rate of neogenesis (15). There is also clear evidence that IGF-2 inhibits cell apoptosis in many cell types. Hill et al. (16) have demonstrated that increased and persistent circulating IGF-2 in neonatal transgenic mice suppresses developmental apoptosis in the pancreatic islets. Conversely, Petrik and al. (17) have suggested that a reduced pancreatic expression of IGF-2 may contribute to the increased apoptosis seen in the fetus after low-protein diet. However, it is unlikely that the reduced pancreatic expression of IGF-2 observed in GK fetuses may contribute to an increased β-cell death because β-cell apoptosis rate was not increased in GK fetuses at late fetal age (3). Due to our observation that the pancreatic expression of IGF-2 is decreased by 55% in GK fetuses, the anomaly of the IGF-2 expression in pancreas of GK fetuses could be the reflection of the reduced β-cell mass (by 60%) observed at this stage. However, in the present study, we observed that the IGF-1 pancreatic expression is similar in GK and Wistar fetuses, and it is known that IGF-1 is produced by fetal and neonatal rat pancreatic islets (18,19). Therefore, the reduced pancreatic IGF-2 expression in GK fetuses cannot be solely attributed to the decreased β-cell mass observed at this stage. Moreover, whereas pancreatic expression of IGF-2 in normal fetus, as shown by in situ hybridization technique, is largely associated with pancreatic endocrine cells (20), it has also been reported within the pancreatic ductal epithelium in neonatal rat pancreas (15).

Recent studies in our laboratory have suggested that the growth and endocrine differentiation of GK and Wistar pancreatic rudiments are identical when followed in vitro (3). Thus, as far as the in vivo fetal situation is recapitulated by the in vitro development of the GK rudiments, the anomaly of the GK rat leading to deficient pancreatic endocrine cell differentiation in vivo could mainly result from a deficiency of one (or several) extrapancreatic factor(s) (3). Because it is known that in the developing normal pancreas, IGF-2 is involved in the regulation of both islet growth and differentiation (5), the reduced circulating IGF-2 levels in GK fetuses could therefore play a crucial role in the anomaly of β-cell mass in the GK fetuses. As circulating levels of IGF-2 in the rat fetus derive predominantly from the hepatic production site (5), the decreased serum IGF-2 levels are probably the result of the decreased IGF-2 mRNA hepatic expression. Involvement of circulating insulin and plasma glucose levels in the regulation of liver IGFBPs production at fetal stages has been repeatedly reported (21), whereas the role of plasma GH on fetal IGF regulation is considered as negligible (10,21). Therefore, it is important to take into account the IGF data available in a rat model of induced (streptozotocin) gestational diabetes with fetal hyperglycemia and hypoinsulinemia in the range of the values found in GK fetuses (10). In this study (10), the fetal serum IGF-2 (and IGF-1) level as well as the fetal liver IGF-2 (and IGF-1) mRNA expression were found to be clearly increased, which is in accordance with reports showing that high glucose in vivo (22) or in vitro (23) increases liver IGF-2 expression. The only in vivo situation with a reported decrease of the serum IGF-2 was...
found in fetuses from undernourished mothers (10). However, in this last situation, both fetal liver IGF-2 (and IGF-1) mRNA levels were still significantly increased as compared with normal fetuses. Taken together, these observations suggest that the situation in the GK fetuses is a very unique one because the decreased IGF-2 level in the serum and its decreased expression in the liver seems to be largely independent of the variations of fetal insulin and glucose levels. Thus, IGF-2 defective production in the GK fetuses may reflect a primary and generalized anomaly. Such a view is also consistent with the results of previous genetic studies reporting that a locus containing the gene encoding the IGF-2 is associated with diabetes phenotype in the GK rat (24). Whereas defective IGF-2 appears to be an early landmark in the pathological sequence leading to retardation of β-cell growth in the fetal GK rat, the pancreatic IGF-2 status in the aging adult GK animal is quite different. Using the swedish GK colony, Höög et al. (25) have reported that an inappropriately processed IGF-2 of high molecular weight accumulates in pancreas from 6-month-old GK rats. More immunoreactive IGF-2 was localized together with insulin to secretory granules in a subset of large and irregular-shaped islets than in either GK rat islets with normal structure or islets from normal rats (26). Because this increase in the high molecular weight form of IGF-2 in the GK pancreas appears to be a late consequence of the early reduction of β-cell number/function in the GK model, it might be triggered by long-term hyperglycemia and might represent an islet self-damaging process disrupting normal arrangement of islet architecture through fibroblast proliferation and collagen deposition.

In view of the reported ability of IGFBPs to modulate IGF bioactivity, we examined serum and tissular expression of IGFBP-1 and -2 in GK fetuses. In general, IGFBP-2 appears to inhibit IGF actions, particularly those of IGF-2 (4). We report here normal serum, liver, and pancreatic mRNA expression of IGFBP-2 in GK fetuses. This conclusion is not at odds with the general agreement that few changes in liver IGFBP-2 mRNA were found in fetuses from experimental diabetic (10) or undernourished mothers (6). By contrast, an increased serum and liver mRNA expression of IGFBP-1 was found in GK fetuses. Insulin appears to play a major role in regulating IGFBP-1 gene transcription in adult animals, i.e., IGFBP-1 transcription is high in diabetic animals and rapidly reduced to normal values after insulin treatment (27,28). The hypoinsulinemic status of GK fetuses could therefore contribute to the high levels of IGFBP-1.

Finally, we have tested the possibility that direct biological action of IGFs on fetal GK β-cell is impaired. Our in vitro results showed that IGF-1 and -2 stimulate the β-cell replication in fetal Wistar islets in accordance with a previous demonstration (29). Similarly, addition of IGF-1 or -2 to the GK isolated islets significantly increased the β-cell replication. These effects were obtained with a submaximal IGF-2 concentration and a maximal IGF-1 concentration based in our circulating-levels evaluation and in vitro data, respectively (19,30). Therefore, we can conclude that IGF responsiveness by fetal GK islets is maintained but cannot eliminate the possibility that the sensitivity of the islet to these factors could be altered.

In conclusion, in GK fetuses at 21.5 dpc, the defective IGF-2 production appears to be an early landmark in the pathological sequence leading to retardation of β-cell growth in the fetal GK rat.

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