

Endocrine Pancreas Development in Growth-Retarded Human Fetuses

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Glucose intolerance in adults born with intrauterine growth retardation (IUGR) may involve peripheral insulin resistance and/or abnormal endocrine pancreas development during fetal life. We quantified insulin-containing cells in deceased human fetuses with IUGR (<10th percentile, $n = 21$) or normal growth (control fetuses, $n = 15$). Paraffin-embedded pancreatic tissues from fetuses older than 32 weeks were obtained from two fetopathology departments. Mean gestational age was 36 weeks in both groups. Tissues with lysis and those fetuses with defects, aneuploidy, or genetic abnormalities were excluded. For each subject, six pancreatic sections spaced evenly throughout the organ were immunostained with anti-insulin antibody. Total tissue and insulin-positive areas were measured by computer-assisted quantitative morphometry. Results were expressed in percentages. To evaluate islet morphogenesis, the percentages of β -cells inside and outside islets were determined. Islet density was similar in the two groups ($P = 0.86$). The percentage of pancreatic area occupied by β -cells (β -cell fraction) was not correlated with gestational age ($r = 0.06$ and $P = 0.97$ in IUGR fetuses; $r = 0.12$ and $P = 0.67$ in control fetuses) or body weight ($r = 0.16$ and $P = 0.47$ in IUGR fetuses; $r = 0.24$ and $P = 0.39$ in control fetuses). Mean β -cell fraction was 2.53% in the IUGR fetuses and 2.86% in the control fetuses ($P = 0.47$). The percentage of β -cells located within islets was identical in the two groups (mean 35%). Our data militate against a primary developmental pancreatic abnormality in human IUGR, leaving peripheral insulin resistance as the most likely mechanism of glucose intolerance in adults born with IUGR. *Diabetes* 51:385–391, 2002

As it was first defined by Lucas (1), “programming” is the development of long-term effects after exposure during a critical period to an endogenous signal or environmental factor. Hales et al. (2) suggested that the association between low birth weight and type 2 diabetes may involve programming, with in utero undernutrition affecting pancreatic β -cell development, thereby leading to impaired β -cell function in adulthood. To our knowledge, this hypothesis is unconfirmed in humans but is strongly supported by animal studies of perinatal undernutrition (3–7). Moreover, intrauterine growth retardation (IUGR) has been associated with a reduction in the number of nephrons in both animals and humans (8,9). However, in a cohort of young adults rigorously selected on birth data, subjects with a history of IUGR had no reduction in insulin secretion, as assessed by oral glucose tolerance tests (OGTTs), but they had hyperinsulinemia (10). This hyperinsulinemia was shown in a later study to reflect true insulin resistance, with appropriate compensatory overproduction of insulin by β -cells (11). The aim of the present study was to investigate whether IUGR is associated with a reduction of β -cell number in the human fetal pancreas. We used quantitative morphometry to measure insulin cell area in pancreatic specimens from eutrophic and growth-retarded fetuses who died in utero.

RESEARCH DESIGN AND METHODS

Cohort selection. All fetuses autopsied between January 1991 and April 1999 in two teaching hospitals after death in utero, at birth, or within 12 h after birth were identified by a retrospective review of 3,363 files. The review was conducted according to French law, and approval of the review procedure by an ad hoc committee was obtained. The parents had given their informed consent to an autopsy for diagnostic and research purposes.

We included only fetuses that died between 32 and 42 weeks' gestational age, which is the period during which intrauterine growth retardation becomes most apparent (12). Fetuses with defects, aneuploidy, or genetic aberrations were excluded, as were fetuses autopsied more than 24 h after death or who had external evidence of maceration suggestive of possible in utero tissue degradation. Availability of paraffin-embedded pancreas specimens was required for study inclusion. Of the 3,363 fetuses, 72 met our criteria and had either IUGR, defined as a body weight <10th percentile for gestational age, or normal growth, defined as a body weight between the 50th and 75th percentiles (13).

Studied population. Figure 1 describes the fetuses with IUGR and control fetuses. Mean gestational age was similar in these two groups (36.8 ± 2.8 and 36.7 ± 3.1 weeks, respectively), as was sex distribution, maternal history, and birth order. Conversely, differences were found in the distribution of causes of death: premature separation of the placenta or fetal distress syndrome caused 76% (16 of 21) of the deaths in the IUGR group and 40% (6 of 15) in the control group. In the control fetuses, 54% of deaths were related to late-pregnancy infections or early neonatal respiratory disease (Table 1). The cause of IUGR

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IUGR, intrauterine growth retardation.

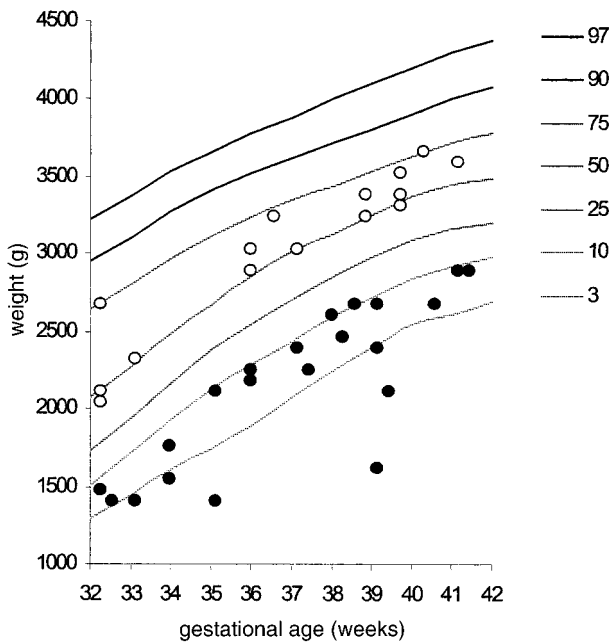


FIG. 1. Weights of the study fetuses according to gestational age plotted on a Leroy-Lefort normative chart showing percentiles in the general population.

was toxemia of pregnancy in 66% of the fetuses (14 of 21). The diagnosis of IUGR caused by toxemia was based on maternal hypertension during pregnancy and/or presence of infarcts in the placenta. One case of IUGR was ascribed to maternal smoking and one to twin pregnancy. In the five remaining cases, the cause of IUGR was unknown.

Pancreatic specimen selection and preservation. The tissues from the study fetuses were examined by a pathologist and graded for tissue lysis according to published criteria (13). Half the fetuses, 31 from the IUGR group and 5 from the normal growth (control group), were excluded from the study because of pancreatic lysis. This left 21 IUGR fetuses and 15 control fetuses with well-preserved pancreatic specimens. Histological examination showed a normal architecture, with preserved exocrine and endocrine structures (Fig. 2a and c). Insulin was detected by immunohistochemistry (Fig. 2b and d) and in situ hybridization (data not shown). Results with these two methods were correlated with each other (data not shown).

Immunohistochemistry. For each of the 36 study fetuses, the entire paraffin-embedded pancreas was cut into 6-µm sections, which were placed on poly-L-lysine-coated slides, dried for 12 h at 37°C, and kept at room temperature. For each pancreas, six sections evenly spaced throughout the organ were selected for immunohistochemistry; in preliminary experiments designed to determine the optimal number of pancreas sections needed to quantify the insulin surface (3–5), we found <6% measurement variability with six sections evenly spaced throughout the pancreas. The paraffin was removed and the sections rehydrated. Antigenic sites were unmasked by treatment for 4 min in 0.01 mol/l citrate buffer (pH 6.0) in a microwave oven. After cooling at room temperature, the sections were washed (Optimax Wash Buffer, Dako). The slides were washed in sterile water and incubated for 10 min with blocking solution (Power Block Universal Blocking Reagent; Biogenex), for 1 h with mouse anti-insulin monoclonal antibody (dilution 1:1,000, Monoclonal

TABLE 1
Causes of death in the two groups

Cause of death	IUGR fetuses	Control fetuses
Premature placental separation	9 (42.8)	5 (33)
Acute fetal distress syndrome	7 (33)	1 (6.6)
Infectious disease during pregnancy	1 (4.7)	4 (27)
Respiratory neonatal disease	2 (9.5)	4 (27)
Unknown	2 (9.5)	1 (6.6)
Total n	21	15

Data are n (%), unless otherwise indicated.

Anti-Insulin; Sigma), for 15 min with a biotinylated complex (dilution 1:40, Multilink Super Sensitive Concentrated; Biogenex), and for 10 min with a streptavidin-coupled alkaline phosphatase solution (dilution 1/40, concentrated enzyme label; Biogenex). Immunoreactivity was revealed using Fast Red as the chromogen (Sigma Fast). The specificity of immunohistochemical staining was checked by omitting either the primary or the secondary antibody or by using only the chromogen. These tests produced no staining.

Computer-assisted quantitative morphometry. Insulin-positive surface area in the sections was quantified using a computer-assisted method. As previously described (3–5,15), we used a microscope (model DMRB; Leica Microsystems, Rueil-Malmaison, France) equipped with a color video camera connected to a Quantimet 500 MC computer (screen magnification ×24). For each section, the total pancreatic tissue area (final magnification ×60) and the insulin-positive area (final magnification ×120) were measured. To evaluate islet morphogenesis, the contributions of intra-islet and extra-islet insulin cells to the total insulin-positive area were determined. An islet was arbitrarily defined as an insulin-positive structure of 50 µm or more in diameter, a size that indicates the presence of ≥10 cells per islet section. The total number of islets was counted, and the result was expressed as the islet number per square millimeter of tissue (islet density). To eliminate interobserver variability in the quantification process, all computer-assisted analyses were done by the same person (F.B.); intraobserver variability was <3%. The results were expressed as the ratio of insulin-positive area to the total tissue area of the section (with β-cell fraction expressed as a percentage), as described previously (5):

$$\beta\text{-cell fraction (\%)} = \frac{\text{Sum of the measured insulin-positive areas on 6 sections}}{\text{Sum of the measured total pancreatic areas on the same 6 sections}} \times 100$$

β-Cell mass could not be precisely calculated for each fetus because the exact pancreatic weight was not known. However, the weight of the dissected pancreatic tissue, including the duodenal part adjacent to the pancreatic head, was not different between the two groups: 3.05 ± 1.15 and 2.75 ± 0.95 g in the control and study fetuses, respectively (P = 0.42).

Statistical analysis. All data were analyzed using Statview (version 5; SAS Institute, Cary, NC). Results are expressed as percentages and means ± SD. The differences between the IUGR and control groups were tested using Student's t test. Correlations between the studied parameters and gestational age or fetal weight were studied using linear regression models. P values ≤0.05 were considered significant. No differences were found between the results obtained on specimens from the two fetopathology departments, and consequently the data from these two departments were pooled for the analyses.

RESULTS

Islet density and β-cell fraction were not related to gestational age or fetal weight. Islet density was not significantly correlated with gestational age (r = 0.16 and P = 0.56 in the control fetuses and r = 0.15 and P = 0.52 in the IUGR fetuses) (Fig. 3) or fetal weight (r = 0.25 and P = 0.38 in the control fetuses and r = 0.092 and P = 0.69 in the IUGR fetuses) (Fig. 3). Consequently, the means in the two groups were compared: mean islet density was not different between the two groups (4.79 ± 2.36 islets/mm² in the control fetuses and 4.66 ± 2.45 islets/mm² in the IUGR fetuses; P = 0.86).

β-Cell fraction was not correlated with gestational age (r = 0.12 and P = 0.67 in the control fetuses and r = 0.06 and P = 0.97 in the IUGR fetuses) (Fig. 3) or fetal weight (r = 0.24 and P = 0.39 in the control fetuses and r = 0.16 and P = 0.47 in the IUGR fetuses) (Fig. 3). We consequently compared the means in the two groups: mean β-cell fraction was 2.86 ± 1.1% in the control fetuses and 2.53 ± 0.9% in the IUGR fetuses (P = 0.47).

No correlation was found between the contribution of intra-islet insulin cells to the total insulin-positive area (intra-islet percent) and gestational age (r = 0.15 and P = 0.59 in the control fetuses and r = 0.24 and P = 0.29 in the IUGR fetuses) (Fig. 3) or fetal weight (r = 0.10 and P = 0.72 in the control fetuses and r = 0.016 and P = 0.94 in the

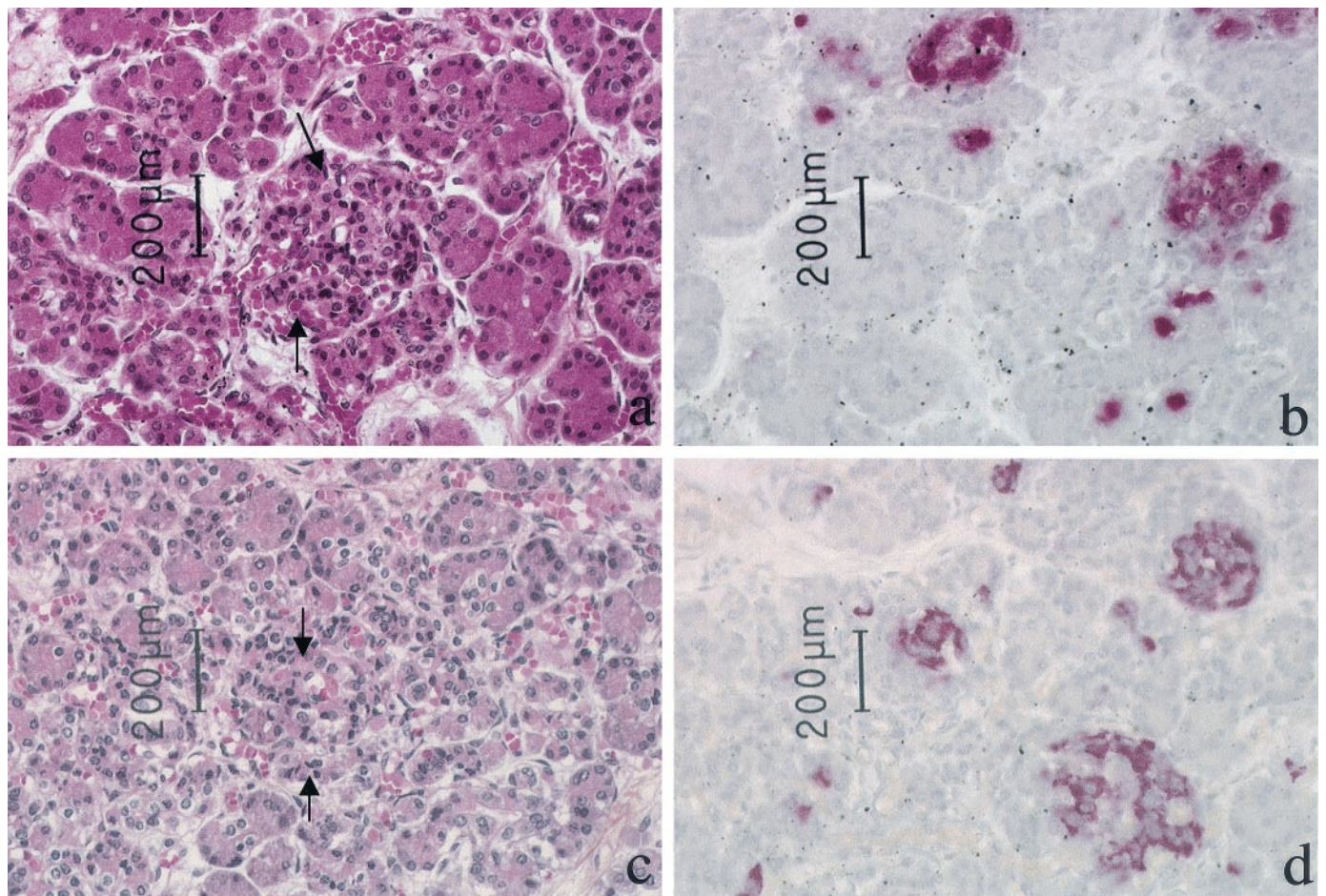


FIG. 2. Histological examination of pancreases from a control (a) and an IUGR fetus (c) showing a normal architecture, with preserved exocrine and endocrine structures (between arrows). Insulin was detected by immunohistochemistry (red color) in pancreases from a control (b) and an IUGR fetus (d).

IUGR fetuses) (Fig. 3). We therefore compared the means in the two groups: mean intra-islet percent was 35% in both groups ($P = 0.71$). Thus, a large number of insulin-producing cells were isolated or in very small clusters in the fetal pancreases examined in our study.

Islet density and insulin-positive areas in study fetuses with vascular IUGR and nonvascular IUGR. Of the 21 fetuses with IUGR, 14 had evidence of vascular disease and 7 did not. Mean gestational age was significantly different between these two subgroups (35.8 ± 2.6 and 39.4 ± 1.5 weeks in the study fetuses with and without vascular disease, respectively; $P = 0.04$). Mean birth weight tended to be lower in the vascular disease group, although the difference was not significant ($2,004 \pm 489$ vs. $2,388 \pm 478$ g, $P = 0.35$). In the vascular disease group, there were nonsignificant trends toward smaller values for β -cell fraction and intra-islet percent (data not shown; β -cell fraction of 2.25 and 2.75% in the subgroups with and without vascular disease, respectively, $P = 0.22$; and an intra-islet percent of 30 and 38%, respectively, $P = 0.19$). A nonsignificant trend toward a difference in islet density was found between the two subgroups (3.88 ± 2.28 and 6.15 ± 2.54 islets/ mm^2 in the subgroups with and without vascular disease, respectively; $P = 0.09$).

DISCUSSION

The exact cause of glucose intolerance in adults born with intrauterine growth retardation is unknown, but it may involve peripheral insulin resistance and/or abnormal pancreatic development during fetal life (16–20). To test the second hypothesis, we quantified insulin-containing cell areas in 21 human fetuses with IUGR and 15 with normal growth.

We worked on a retrospective cohort of autopsied human fetuses and newborns for whom pancreatic specimens were available (21). We selected well-preserved pancreatic specimens for our studies. Tissue preservation was checked using histological examination, immunohistochemistry, and in situ hybridization. The two last tests produced well-correlated results and allowed us to evaluate insulin protein and mRNA expression in our specimens. Moreover, our findings in the control fetuses (2.86% mean β -cell fraction) were comparable to those obtained by others working on smaller numbers of specimens. Van Assche et al. (22) found a β -cell fraction of 2.1% in a study of human pancreas specimens from 10 fetuses of 28–32 weeks' gestational age, and Rahier et al. (23) found fractions of 4.7% in six specimens from human newborns aged 4–15 days.

In our study of pancreases from fetuses aged 32–42

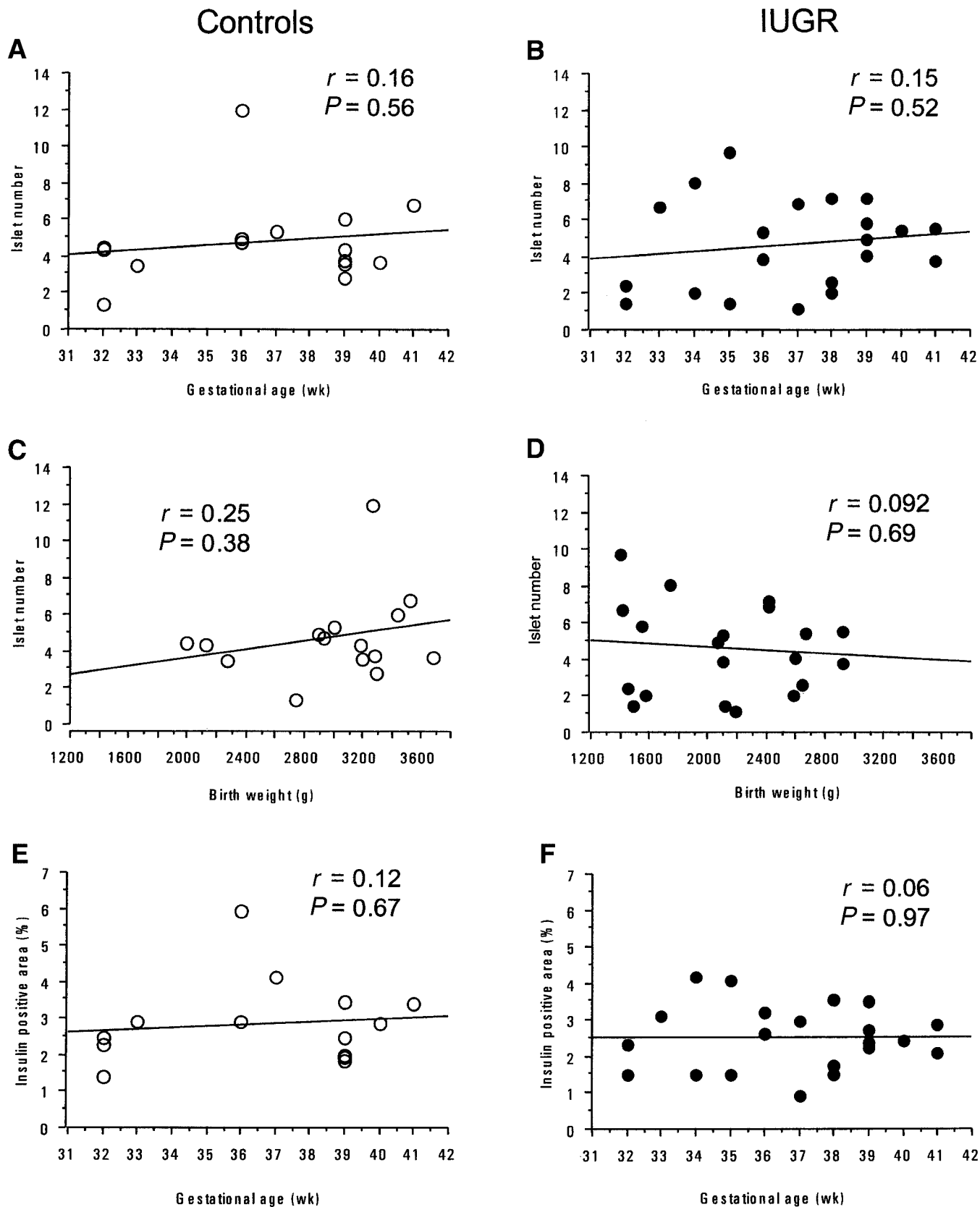


FIG. 3. Relationship between morphometric characteristics and gestational age or fetal weight in IUGR (B, D, F, H, J, and L) and control (A, C, E, G, I, and K) fetuses. Islet number (A–D) is expressed by square millimeter of tissue (islet density), insulin-positive area (E–H) is expressed as the ratio of insulin-positive area to the total tissue area of the section (with β -cell fraction expressed as a percentage), and insulin-positive area in islets (I–L) is expressed as the percentage of the total insulin-positive area located within islets, as defined in the RESEARCH DESIGN AND METHODS section.

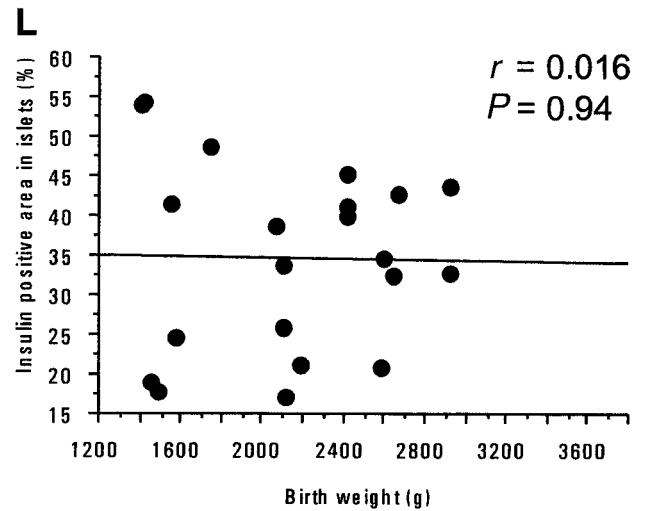
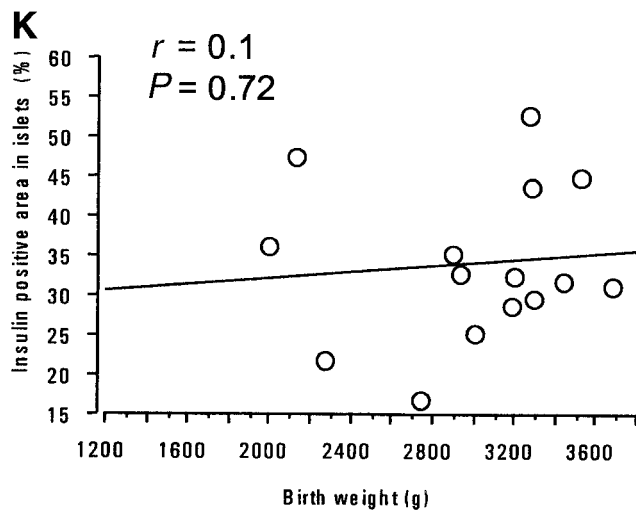
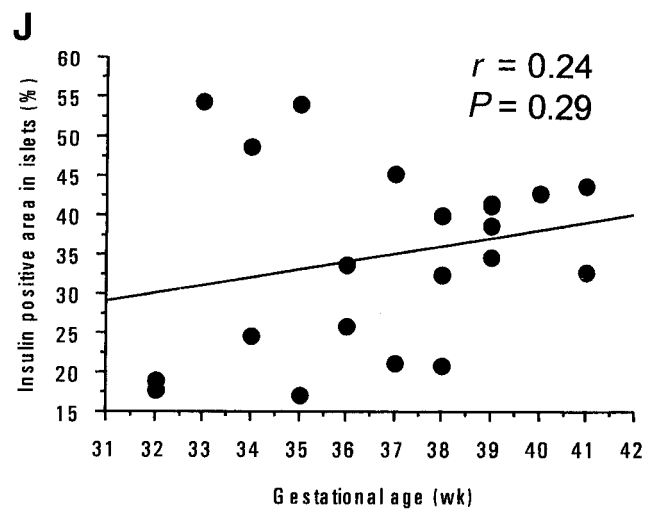
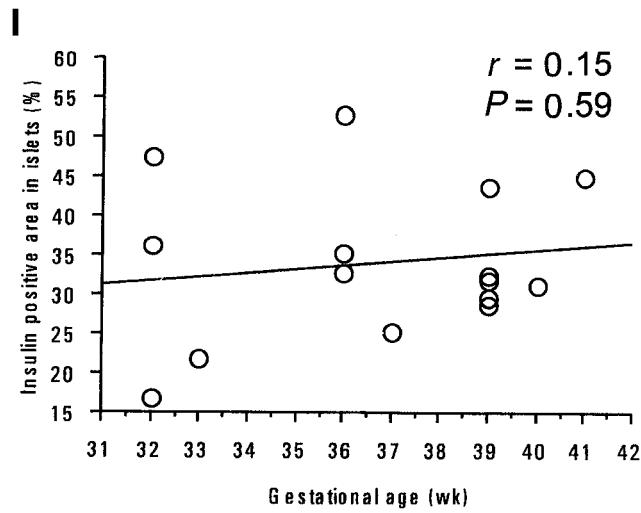
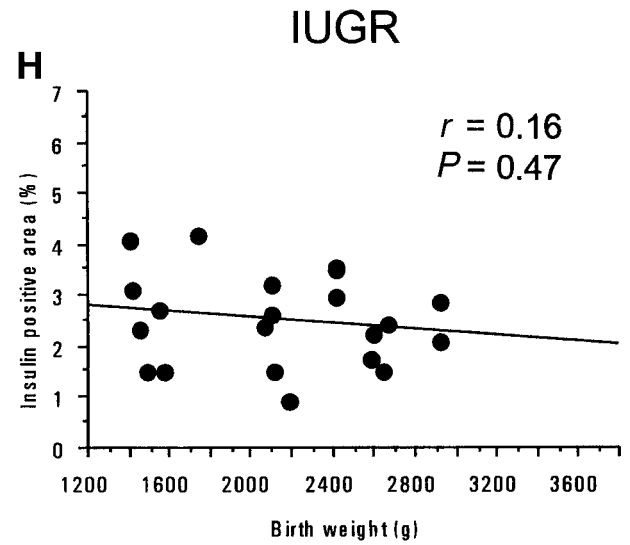
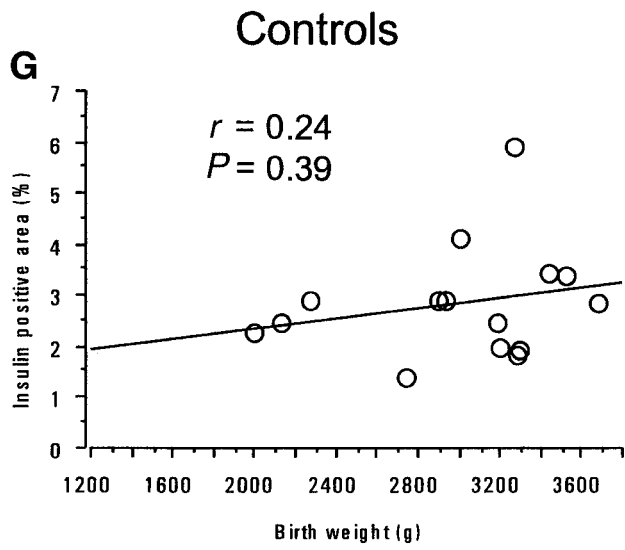


FIG. 3—Continued

weeks, we found no major changes with gestational age in β -cell fraction, islet density, or percentage of insulin cells located within islets (intra-islet percent) in the control fetuses, nor did we find any differences in β -cell fraction or islet organization between IUGR fetuses and control fetuses during the last 2 months of pregnancy. In our cohort, the pancreases were not weighed in a standardized manner. Consequently, we expressed our results as percentages rather than absolute β -cell mass. However, because no significant differences in the dissected duodenopancreatic tissue weight were found between the IUGR and control fetuses, it is unlikely that significant differences would exist in the pancreatic weight between those groups. Therefore, it is unlikely that there was a reduction in the absolute β -cell mass of the IUGR fetuses due to relatively smaller pancreas.

A striking finding from our study is the large number of insulin-producing cells that were isolated or grouped in small clusters. This finding is in line with previous studies of the human fetal (24,25) or adult (26) pancreas. Isolated insulin-producing cells, some of which are very near the ducts, may be newly differentiated cells that will later organize into islets. Intra-islet percent was identical in IUGR and control fetuses, a finding that militates against abnormalities in islet morphogenesis in the IUGR fetuses. Nevertheless morphogenesis of the islets was not yet finished by the time the fetuses were analyzed. This leaves the possibility open that a defect in morphogenesis may be present in the neonatal period or that altered pancreatic plasticity to adapt β -cell mass to situations of increased insulin demand could develop later in life.

The results of this experimental work do not provide information on β -cell function in fetuses with IUGR. Several studies have addressed this question. In a study conducted by our group, no significant differences in OGTT-derived insulin secretion indexes determined at 20 years of age were found between subjects born with IUGR and control subjects (10). In another study, we showed that first-phase insulin release during intravenous glucose tolerance testing was inversely related to insulin sensitivity but was not independently influenced by a history of IUGR (11): the values in subjects born with IUGR were comparable to those described previously in healthy individuals with normal glucose tolerance. These data strongly suggest that individuals born with IUGR can compensate for their decreased insulin sensitivity by increasing their insulin secretion. In a study of intravenous glucose tolerance tests in young adults, Flanagan et al. (27) showed that low birth weight was associated with reduced insulin sensitivity and increased insulin secretion. In a recent hyperglycemic clamp study, no deficiency in insulin and/or C-peptide secretion was found in insulin-resistant subjects born with IUGR as compared with control subjects (28). Thus, individuals born with IUGR can maintain an adequate insulin response to sustained glucose stimulation.

In contrast to results in humans, findings from animal models strongly suggest that poor nutrition during fetal life may impair β -cell development, leading to β -cell dysfunction in late adulthood. Perinatal undernutrition affected islet neogenesis and permanently impaired β -cell mass in young adult rats (3–5). Feeding the dams a low-protein diet reduced neonatal β -cell proliferation and

impaired glucose tolerance in the offspring during early adulthood (6). These discrepancies between results in humans and animals indicate a need for caution in extrapolating animal data to humans. Available data suggest that in utero undernutrition in humans may be associated with insulin-resistance but not with abnormal β -cell development or β -cell dysfunction.

In conclusion, we found no differences between IUGR and control fetuses in insulin-positive area or islet organization during the last 2 months of pregnancy. Our data are in accordance with earlier studies showing that insulin secretion is adapted to insulin resistance in human adults born with IUGR. They do not support a role for pancreatic abnormalities in the development of glucose intolerance in adulthood.

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