Role of Apoptosis in Failure of β-Cell Mass Compensation for Insulin Resistance and β-Cell Defects in the Male Zucker Diabetic Fatty Rat

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To define the mechanisms involved in the evolution of diabetes in the Zucker diabetic fatty (ZDF) rat, β-cell mass and replication rates were determined by immunochemistry, point-counting morphometry, and 6-h 5-bromo-2′-deoxyuridine (BrdU) incorporation. The β-cell mass in 5- to 7-week-old prediabetic ZDF rats (4.3 ± 0.06 mg) was similar to age-matched insulin-resistant Zucker fatty (ZF) rats (3.7 ± 0.05 mg) and greater than that in Zucker lean control (ZLC) rats (1.9 ± 0.3, $P < 0.05$). At 12 weeks (after diabetes onset), β-cell mass in the ZDF rats (8.1 ± 1.7 mg) was significantly lower than the ZF rats (15.7 ± 1.8 mg). The mass in the ZF rats was significantly greater than in the ZLC rats (4.3 ± 0.8 mg, $P < 0.05$). The β-cell proliferation rate (mean of both time points) was significantly greater in the ZDF rats (0.88 ± 0.1%) compared with the ZF and ZLC rats (0.53 ± 0.07%, 0.62 ± 0.07%, respectively, $P < 0.05$), yet ZDF rats have a lower β-cell mass than the ZF rats despite a higher proliferative rate. Morphological evidence of neogenesis and apoptosis is evident in the ZF and ZDF rats. In addition, even at 5–7 weeks a modest defect in insulin secretion per β-cell unit was found by pancreas perfusion. These studies provide evidence that the expansion of β-cell mass in response to insulin resistance and insulin secretory defects in diabetic ZDF rats is inadequate. This failure of β-cell mass expansion in the ZDF rat does not appear to be from a reduction in the rate of β-cell proliferation or neogenesis, suggesting an increased rate of cell death by apoptosis. *Diabetes* 47:358–364, 1998

Human autopsy studies have suggested that β-cell mass is only ~50% in patients with obese NIDDM compared with obese nondiabetic control patients (1–3). This inadequate β-cell mass may be an important component of the abnormal β-cell function that occurs in NIDDM. However, prospective data on β-cell mass kinetics during the evolution of NIDDM are not available, and it is not currently known whether reduced β-cell mass in NIDDM is due to an increased loss of β-cells and/or to inadequate β-cell expansion to compensate for insulin resistance and β-cell secretory defects.

The present study was undertaken to address this question in an animal model of NIDDM, the Zucker diabetic fatty (ZDF) rat. This model was selected because of the many similarities to human NIDDM. Homozygous male inbred Zucker diabetic fatty (ZDF/GmiTM-*fa/fa*) rats all develop diabetes around 10 weeks of age after a prediabetic period during which obesity and insulin resistance are present, but blood glucose concentrations (as assessed by intraperitoneal glucose tolerance testing [IPGTT]) are normal (4–6). Obesity in these animals is due to mutations in the leptin receptor (7,8). Results in prediabetic and diabetic ZDF rats were compared with two groups of controls: Zucker lean control (ZLC) nondiabetic male littermates (ZLC +/*fa* or +/+) as well as with male Zucker fatty *fa/fa* rats (9). The Zucker fatty rat, a partially outbred strain, has the same mutation in the leptin receptor as the ZDF rats and is therefore also obese and insulin resistant. However, in contrast to the ZDF model, glucose concentrations in these animals are normal or reflect only mild glucose intolerance at age-matched time points. Comparison of results in the Zucker fatty *fa/fa* and ZDF rats discriminates the effects of diabetes from the effects of insulin resistance and/or obesity. In the present study, β-cell mass and β-cell replication rates were measured at 5–7 and 12 weeks of age, the prediabetic stage and after the onset of overt diabetes, respectively. The study of the evolution of glucose intolerance in this model from the prediabetic phase to overt diabetes provides insights into the pathophysiological factors responsible for the changes in β-cell mass as diabetes develops. Quantitation of β-cell replication rates allowed the dynamics of β-cell turnover to be modeled.

In addition, insulin secretion data obtained from isolated perfused pancreas experiments were analyzed after correction for changes in β-cell mass to provide information on insulin secretion per unit β-cell mass.

RESEARCH DESIGN AND METHODS

Animals. Male Zucker diabetic fatty (ZDF/GmiTM *fa/fa*) rats were studied at 5–7 (prediabetic phase) and 12 (overt diabetic phase) weeks. Age-matched Zucker fatty *fa/fa* and Zucker lean control rats (GmiTM +/*fa* or +/+) were used as controls. The precise time of study at the 5–7 week time point was 46 days for the lean control rats, 38 days for the fatty rats, and 43 days for the ZDF rats. All three groups were studied at 84 days at the 12-week time point. The lean controls are littermates of the ZDF animals and are either heterozygous for, or lack, the ZDF/(*fa/fa*) mutation. The lean control animals were not genotyped as these animals were studied prior to the discovery of the leptin gene. The ZDF rats and lean littermates were purchased from Genetic Models (Indianapolis, IN), and the Zucker fatty rats

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BrdU, 5-bromo-2′-deoxyuridine; DAB, diaminobenzadine; IgG, immunoglobulin G; IPGTT, intraperitoneal glucose tolerance test; PBS, phosphate-buffered saline; ZDF, Zucker diabetic fatty; ZF, Zucker fatty; ZLC, Zucker lean control.

were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Animals were maintained on an ad libitum diet with commercial chow (Purina 5008; 6.5% fat) and had free access to water. All institutional guidelines for care and use of animals were followed.

Animals used for morphometric studies received intraperitoneal injections of 5-bromo-2′-deoxyuridine (BrdU; Sigma, St. Louis, MO), 100 mg/kg body wt 6 h prior to being killed. Pancreases were obtained from 5- to 7- and 12-week-old ZDF, ZF, and ZLC rats, for the measurement of β-cell mass. After removal from the animals, the organs were cleared of fat and lymph nodes, weighed, fixed in Bouin's solution, and embedded in paraffin. The pancreases were divided into two (5–7 weeks) or three (12 weeks) blocks.

Glucose tolerance testing. After an overnight fast (~15 h), 2 g/kg glucose was administered intraperitoneally to conscious rats. Blood was obtained from the tail before and 60 min after the administration of glucose and was analyzed for glucose with the Hemacue B glucose photometer (Hemocue AB, Angelholm, Sweden). Food was removed at ~5:30 P.M., and studies were commenced at ~8:30 A.M. the next morning (~15 h). Standard light/dark cycles were maintained by the animal facility with lights on from 6:00 A.M. to 6:00 P.M. and lights off for the remaining 12 h.

Measurement of β-cell mass. β-cell mass was measured by point-counting morphometry of insulin immunostained pancreatic sections (3–5 μm) as described by Weibel (10) and as applied by Bonner-Weir (11) to endocrine pancreas. A polyclonal guinea pig anti-porcine insulin antibody was used. Briefly, sections were incubated with primary antibody at 1:300 dilution, for 1 h at room temperature or at 4°C overnight and immunoperoxidase labeled with the Vectastain system (Vectastain ABC kit), developed with 3,3′ diaminobenzidine (DAB, Sigma Immunochemicals), and counterstained with hematoxylin. An Olympus BH2 microscope connected to a Sony CCD color camera and Sony color monitor with a 48-point transparent overlay was used for point counting. One random section of each block was scored systematically at a final magnification of 406×. In nonoverlapping fields, the number of intercepts over β-cell, endocrine non–β-cell, exocrine pancreatic tissue, and non–pancreatic tissue was determined. A minimum of 200 fields per animal was counted. Using the nomogram given by Weibel (10) and the relative volumes measured, the expected relative probable error in percentage of mean of the relative volume should be 15% for ZLC, 7% for ZF, and 10% for ZDF. This relative probable error is similar to the coefficient of variance. All sections were blinded before quantitation and read by one observer (A.P.).

Measurement of β-cell replication rates and average cell size. Other sections from the blocks used for β-cell mass measurement were used to study β-cell replication rates (11). BrdU is incorporated into newly synthesized DNA and therefore labels replicating cells (12). A 6-h incorporation interval was chosen as this is the duration of the rat G_2+M phase (13) and avoids the possibility of inclusion of daughter cells. Sections (3–5 μm) were double-stained with immunoperoxidase for BrdU and for islet endocrine non–β-cells. A cell proliferation kit was used for immunostaining BrdU (Amersham International, Amersham, U.K.). Briefly, sections were incubated with a mouse anti-BrdU monoclonal antibody for 30 min at room temperature, washed with phosphate-buffered saline (PBS; pH 7.4), incubated with an affinity-purified peroxidase antimouse immunoglobulin G (IgG), and stained with diaminobenzadine (DAB) plus a substrate/intensifier containing hydrogen peroxide and nickel chloride/cobalt chloride.

The sections were then stained for the endocrine non–β-cells with varying dilutions of a mixture of rabbit antibodies against pancreatic polypeptide, somatostatin, and glucagon obtained from Linco (St. Charles, MO). After an overnight incubation at 4°C, the sections were peroxidase labeled with the Vectastain ABC system, developed with DAB, and counterstained with hematoxylin. On these stained sections, the endocrine non–β-cells have orange-brown cytosol, the core of β-cells have nonimmunostained cytosol, and $BrdU^+$ cells appear with blue-black nuclei. In the case of the islets with disrupted non–β-cell mantles, cells within the islets with characteristic large, round nuclei and significant surrounding cytoplasm that did not stain for glucagon, somatostatin, or pancreatic polypeptide were regarded as β-cells. The entire section, or 1,500 β-cell nuclei, were systematically counted. All sections are blinded at the time of quantitation. The proportion of $BrdU^+$ β-cell nuclei to total β-cell nuclei was calculated and represents the percentage β-cell replicative rate per 6-h time interval.

Average cell size was measured using the above sections from 5- to 7-week-old and 12-week-old ZDF rats (n = 5 per group). Islet area was measured with Image-Pro plus quantitative software (Version 3.0 for Windows 95/NT, Silver Spring, MD), and 796.7 ± 11.8 β-cell nuclei per animal were sampled. The average cell size was determined by dividing the measured islet area by the number of β-cell nuclei within each islet area sampled.

Apoptosis detection by propidium iodide staining. Tissue sections (3 μm) were cut from the tail blocks of the 5- to 7-week-old rats. ZLC and ZDF rats were immunostained for insulin and stained with propidium iodide (14,15). Propidium iodide is a fluorescent dye that binds to nucleic acids (and therefore all nuclei) thereby allowing the more intensely fluorescent and condensed or fragmented nuclei characteristic of apoptotic cells to be identified. Sections were deparaffinized, rehydrated, and immunostained for insulin, as described above, and then incubated for 10 min with propidium iodide 10 μg/ml and RNAse A 100 μg/ml at 37°C. Sections were then washed extensively with PBS, rinsed with distilled water, and mounted with an aqueous media (AFT, Behring Diagnostics, Sommerville, NJ). On a Zeiss LSM confocal microscope, islet tissue was identified under bright field, and then using a rhodamine filter set, islet nuclei were examined. Cells with condensed or fragmented nuclei characteristic of apoptosis can be distinguished from pattern of heterochromatin seen in most cells in these fixed tissues.

Mathematical modeling of the dynamic changes in β-cell mass. Dynamic changes in β-cell mass were modeled based on the following modified mass balance equation (16):

$$d(\beta\text{-cell mass})/dt = \{[\text{replication} + (\text{neogenesis–death})] \times \beta\text{-cell mass}\}$$

where d(β-cell mass)/dt is the change in β-cell mass over the time interval dt. Replication, neogenesis, and death refer to the relative rates, per 6 h, of each of these processes. Multiplication of the term [replication + (neogenesis–death)] by the term β-cell mass converts these relative rates to an absolute value for the change in β-cell mass over time.

This equation assumes that there is no change in average cell size, so change in β-cell mass directly reflects change in cell number. The average cell size in the ZDF rats at 5–7 weeks (131.1 ± 4.29 μm^2) was not significantly different from the average cell size in ZDF rats at 12 weeks (134.3 ± 3.4 μm^2), substantiating the validity of the equation used.

In the present studies, rates of β-cell neogenesis and death were not measured independently. There is no method currently available to quantify neogenesis, and the low frequency of morphological evidence of apoptosis in tissue sections makes quantitation very difficult. However, since β-cell mass and proliferative rates were measured at two discrete time points, it is possible to solve the mass balance equation provided above to derive the combined (neogenesis–death) term and gain insight into the net effect of the balance between these two opposing processes as follows:

In considering the variables contained in the mass balance equation, β-cell mass for each animal group was measured at 5–7 and 12 weeks, defining dt as the time interval between these two experimental sampling times, and the replication rate was obtained from the average β-cell BrdU incorporation rate over the time interval between 5–7 and 12 weeks.

$$\beta\text{-cell mass at 12 weeks} = \beta\text{-cell mass at 5–7 weeks} \times \{1 + [\text{replication} + (\text{neogenesis–death})]\}^{\Delta\text{days} \times 4}$$

This equation was solved using β-cell mass and replication rate measurements at 5–7 and 12 weeks. Δdays is multiplied by 4 since the relative rates are per 6 h and not per day.

Using the measured values of β-cell mass at 5–7 and 12 weeks and the replication rate calculated as described above in the equation, we are able to solve for the (neogenesis–death) term.

If the calculated (neogenesis–death) term is negative, this implies that the rate of β-cell death exceeds the rate of β-cell neogenesis; i.e., the measured β-cell mass at 12 weeks is less than the β-cell mass at 12 weeks estimated from the measured β-cell replication rate and from the β-cell mass measured at 5–7 weeks. Conversely, a positive (neogenesis–death) term implies that the rate of β-cell neogenesis exceeds the rate of β-cell death, and the measured β-cell mass at 12 weeks is more than expected based on the measured β-cell mass at 5–7 weeks and β-cell replication rate. Please note that this modeling approach does not distinguish between a fall in rate of neogenesis and an increase in rate of cell death.

Insulin secretion from the isolated perfused pancreas. To study changes in insulin secretion in relation to changes in β-cell mass, insulin secretion rates were obtained using the isolated perfused pancreas from 5- to 7-week-old ZLC, ZF, and ZDF rats as previously described (17). These animals are different from those used to study β-cell mass. The ZLC animals (n = 11) were studied at 49 ± 1.8 days, the ZF animals (n = 13) at 47.8 ± 0.8 days, and the ZDF rats at 47.2 ± 1.2 days (n = 10).

Insulin secretion was measured in response to a progressive increase in the perfusate glucose from 2 to 20 mmol/l over a period of 90 min. Insulin concentrations were measured in duplicate by a double antibody radioimmunoassay using a rat insulin standard. The intra-assay coefficient of variation was 8%.

Total insulin secretory output from each pancreas was measured and expressed as μU/ml. In addition, the secretion data were reanalyzed in each animal group adjusting for the measured differences in β-cell mass. Each data point was divided by the measured β-cell mass in milligrams for that particular animal group. The insulin secretory output represented in this fashion gives a reflection of β-cell function per unit β-cell mass.

Statistical analysis. Results are expressed as means ± SE. The statistical significance of differences between the three groups of rats at 5–7 and 12 weeks was

determined by one-way analysis of variance with Tukey's adjustment for post hoc comparisons. A P value of <0.05 was considered significant. Between-group comparisons at 12 weeks were made as above but performed with a log transformation of the data to normalize the variance of the measurements between groups and over a range of responses. Statistical tests were performed using the Statistical Analysis System (SAS for Windows V6.11; SAS, Cary, NC).

RESULTS

Animal weight and glucose tolerance. ZDF and ZF rats were significantly heavier than the lean controls at both ages as shown in Table 1. At 5–7 weeks, the fasting glucose levels in both the ZF and ZDF rats were significantly higher than in the lean animals ($P < 0.05$), and at 60 min after the glucose challenge were significantly greater in the ZDF compared with the ZF and lean rats ($P < 0.05$). At 12 weeks, the fasting glucose was significantly elevated in the ZDF animals compared with the ZF and lean animals ($P < 0.05$), and at 60 min after the glucose challenge, the glucose value in the ZDF and fatty rats was significantly greater than that in the lean rats ($P < 0.05$).

Islet morphology. The Zucker lean control animals have normal islet architecture at both 5–7 and 12 weeks of age. The islets are round or oval, with a clear mantle of non–β endocrine (glucagon, pancreatic polypeptide, somatostatin) cells and a core of uniformly intensely insulin-stained β-cells (Fig. 1*A*,*D*).

In contrast, Zucker fatty rats have large elongated and often bilobed islets, suggesting islet hyperplasia; these changes are more marked at 12 weeks. Occasional fibrotic, irregular islets are seen. Insulin staining is usually uniform (Fig. 1*B*). The non-β endocrine cell mantle is usually intact, but occasional irregularly shaped islets are seen in which the mantle is disrupted with non-β endocrine cells appearing more centrally located and scattered throughout the islet (18–20) (Fig. 1*E*).

Islets from the 5- to 7-week-old ZDF rats appear similar to the age-matched ZF animals, except abnormal irregular, fibrotic islets are slightly more frequent. By 12 weeks, many ZDF islets are markedly abnormal with fibrosis and irregular projections into the exocrine tissue. The insulin staining is of variable intensity, suggesting evidence of insulin degranulation (Fig. 1*C*). In both ZDF and ZF animals, small ductules can be seen within the irregular islets.

In both groups, more noticeably in the ZF rats, small islets with intense insulin-staining and few (or none) non-β endocrine cells are seen throughout the pancreas. At 5–7 weeks, the non-β endocrine cell mantle is normal in ZDF islets. By 12 weeks, however, the mantle is disrupted with non-β endocrine cells scattered throughout the islet, even in islets that maintain a normal shape (Fig. 1*F*).

In pancreases from ZDF and ZF rats at both 5–7 and 12 weeks, multiple (often >20) $BrdU^+$ cells are evident in the epithelium of common pancreatic ducts as well as in the main and interlobular ducts. Such cells are occasionally seen in the lean control animals but are noticeably less numerous (Fig. 2*B*). In the ZDF and ZF animals, small clumps of cells that stain for insulin or non–β-cell hormones are seen budding from the ducts (Fig. 2*A*). These features suggest active neogenesis, the formation of new islets by differentiation of ductal precursor cells (21,22).

Pancreatic and β-cell mass. β-cell mass increased in the ZF rats from 3.7 ± 0.05 mg at 5–7 weeks to 15.7 ± 1.8 mg at 12 weeks (4.2-fold increase). The corresponding values for β-cell mass at the same time points were 4.3 ± 0.06 mg to 8.1 ± 1.7 mg in the ZDF rats (1.9-fold increase) and 1.9 ± 0.3 mg to 4.3 ± 0.08 mg in the lean controls (2.3-fold increase).

At 5–7 weeks, the β-cell mass in both ZDF and ZF rats was approximately double (2.2-fold and 1.9-fold, respectively, $P < 0.05$, by analysis of variance [ANOVA]) than that in Zucker lean controls (Table 2), but did not differ from each other. At 12 weeks, β-cell mass in the ZDF and ZF rat was significantly greater than that of the lean controls ($P < 0.05$), with that of ZDF increased by 1.9-fold, and that of ZF by 3.7-fold, compared with the lean animals. Differences in β-cell mass in the 12-week animals analyzed using a log transformation of the data shows that the differences between ZLC and ZF and between ZF and ZDF rats were significant ($P < 0.05$). The difference in mean β-cell mass between the 12-week-old lean and ZDF rats, however, did not reach statistical significance.

β-cell replication rates. Average rate of β-cell proliferation in each animal group was calculated as the mean of the values at each of the two time points measured by bromodeoxyuridine incorporation over a 6-h interval (Table 2.). The β-cell replication rate in the ZDF rat (0.88 ± 0.10%) is significantly greater than that of either the ZLC control (0.53 ± 0.07%) or ZF (0.62 ± 0.07%) animals ($P < 0.05$).

Mathematical modeling of the dynamic changes in β-cell mass. In the lean control animals, the measured β-cell mass at 12 weeks was 4.3 ± 0.8 mg and the estimated β-cell mass calculated by using the average replication rate and the measured β-cell mass at 5–7 weeks was 4.23 mg. This close concordance between the measured and estimated β-cell mass values implies that the rate of β-cell death is nearly equal to the rate of β-cell neogenesis. The value of the (neogenesis–death) term is 0.01%.

In the ZF rats, the measured β-cell mass at 12 weeks is 15.7 ± 1.8 mg, and the estimated value is 11.91 mg. This difference with a (neogenesis–death) term of 0.148% indicates that between 5–7 and 12 weeks, the rate of β-cell neogenesis exceeds the rate of β-cell death.

TABLE 1
Changes in glucose tolerance and body mass in the three groups of male Zucker rats at 5–7 and 12 weeks

	Glucose (mg/dl)					
	0 min		60 min		Body mass (g)	
	5–7 weeks	12 weeks	5–7 weeks	12 weeks	5–7 weeks	12 weeks
ZLC	86 ± 6	88 ± 8	124 ± 10	167 ± 9	133 ± 6	293 ± 3
ZF	109 ± 4*	86 ± 2	136 ± 11	298 ± 41*	166 ± 2*	438 ± 11*
ZDF	101 ± 3*	131 ± 2*	196 ± 13*	369 ± 6*	159 ± 11*	383 ± 3*

Data are means ± SE. IPGTT was performed as described in METHODS.(5–7 weeks n = 4–6, 12 weeks n = 5–7). *$P < 0.05$.

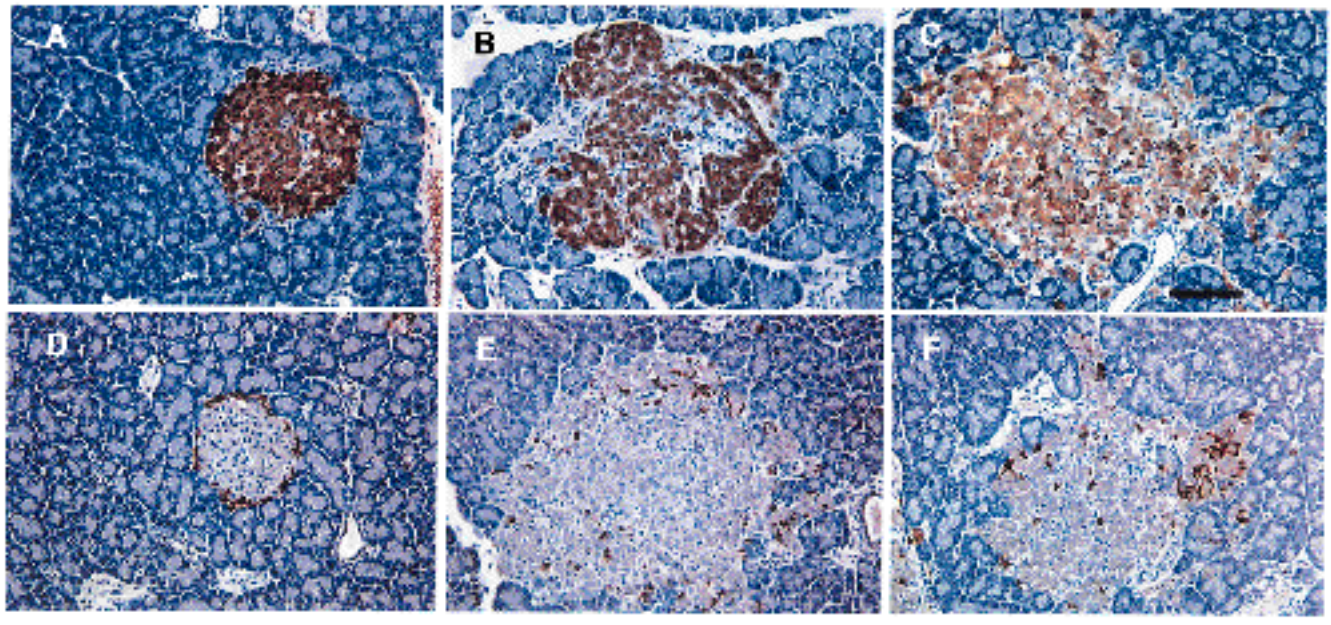

FIG. 1. Islet morphology of 12-week-old male Zucker lean control (*A*,*D*), Zucker fatty (*B*,*E*), and Zucker diabetic fatty (*C*,*F*) rats as shown by immunoperoxidase (brown) staining for insulin (*A*–*C*) and for the non–β-cell hormones (glucagon, somatostatin, and pancreatic polypeptide) (*D*–*F*). In comparison to the normal mantle of non–β-cells around a core of β-cells (*A*,*D*), the islets of Zucker fatty (*B*,*E*) tend to be larger, with some disruption of the mantle/core arrangement; some irregularity of the islet shape is apparent. The ZDF islets (*C*,*F*) are similar to the Zucker fatty islets, except that the β-cells are partially degranulated and tend to be more irregular in shape. Hematoxylin counterstained. Magnification bar = 100 μm.

In contrast, measured β-cell mass in the ZDF rats was 8.1 ± 1.7 mg at 12 weeks, and the estimated β-cell mass was 18.15 mg; hence, the (neogenesis–death) term is –0.495%, indicating that the rate of β-cell death exceeds the rate of new β-cell formation by neogenesis between 5–7 and 12 weeks. This could be due to a marked decrease in neogenesis or an increase in cell death. Since neogenesis was observed in ZDF as well as ZF rats, the mathematical modeling suggests a marked increase in cell death in the ZDF rat.

Apoptosis detection. Apoptosis is a likely mechanism mediating the increase in cell death suggested by the kinetic analysis presented above. Sections used for the morphometric analysis were examined for morphological evidence of apoptosis (23–25). Sections from the 5- to 7-week-old ZDF animals had evidence of β-cell apoptosis. To characterize this further, pancreatic sections from the 5- to 7-week-old ZDF and lean animals were double-stained for insulin and propidium iodide. In 5- to 7-week-old ZDF animals, several clear cut apoptotic β-cells in each section were seen, whereas only a few apoptotic exocrine cells were seen in ZLC rats. However, the low frequency of apoptotic β-cells precludes meaningful quantification.

Perfused pancreas insulin secretion adjusted for differences in β-cell mass. Figure 3 shows insulin secretion at 5–7 weeks in the three animal groups expressed either as total insulin output (Fig. 3*A*) or per unit β-cell mass (Fig. 3*B*).

Mean total insulin secretory output from the perfused pancreas as glucose levels were increased from 2 to 20 mmol/l was 233.7 ± 70.5 μU/ml in the ZLC animals, 482.6 ± 133.9 μU/ml in the ZF animals, and 420.7 ± 97 μU/ml in the ZDF rats. Insulin secretion was significantly greater in the ZDF and ZF rats, compared with the lean controls ($P < 0.05$). When analyzed by

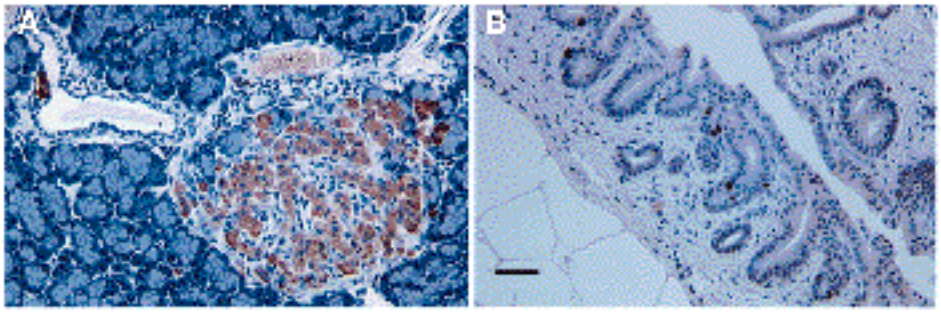

FIG. 2. In both Zucker fatty and Zucker diabetic fatty pancreases, there is evidence of neogenesis. A small clump of cells immunostained for insulin (brown) can be seen budding from the duct in the upper left (*A*). Nuclei incorporating BrdU (black) are found in the epithelium of the common pancreatic duct (*B*). Hematoxylin counterstained. Magnification bar = 50 μm.

TABLE 2
Changes in pancreatic mass, β-cell mass, and β-cell replication rates in the three groups of male Zucker rats at 5–7 and 12 weeks

	Pancreas mass (g)		β-cell mass (mg)		% β-cell BrdU$^+$/ 6 h	
	5–7 weeks	12 weeks	5–7 weeks	12 weeks	5–7 weeks	12 weeks
ZLC	0.73 ± 0.02	1.31 ± 0.05	1.9 ± 0.3	4.3 ± 0.8	0.56 ± 0.14	0.50 ± 0.07
ZF	0.63 ± 0.04	1.13 ± 0.07	3.7 ± 0.05*	15.7 ± 1.8*	0.52 ± 0.04	0.68 ± 0.10
ZDF	0.53 ± 0.03	1.13 ± 0.01	4. 3 ± 0.06*	8.1 ± 1.7†	0.85 ± 0.13*	0.92 ± 0.15*

Data are means ± SE. $P < 0.05$ vs. lean control, *$P < 0.05$ vs. lean control using mean of each of the two time points (5–7 weeks n = 4–6, 12 weeks n = 5–7), †ZDF significantly different from ZF (ANOVA with log transformation $P < 0.05$).

ANOVA, insulin secretory output adjusted for β-cell mass was not significantly different between the three animal groups. However, insulin secretion from the perfused pancreas of one ZDF rat was a clear outlier, as judged by Dixon's gap test ($P < 0.005$), with insulin secretion rates threefold greater than in the other animals. Data from this animal was therefore excluded from further analysis. Pancreatic insulin secretion per unit β-cell mass was compared in the ZDF and ZF animals with the lean control animals. Insulin secretory output per unit β-cell mass was significantly greater in the ZF than in the ZDF rats ($P = 0.024$ using the Z test), providing further evidence of a functional defect already present in the ZDF (6,17).

DISCUSSION

Insulin resistance and β-cell dysfunction are the two predominant and characteristic features of NIDDM. In the evolution of this condition, insulin resistance is usually identifiable before β-cell dysfunction can be detected by clinical and physiological testing (26). Insulin-resistant subjects maintain normal glucose tolerance by adaptive hypersecretion of insulin, thereby compensating for the reduction in insulin action. In certain individuals, these compensatory mechanisms become inadequate with time, and overt hyperglycemia and clinical diabetes supervenes (27). The nature of the mechanisms in the β-cell that compensate for insulin resistance and the reasons for their failure are thus key to our understanding of the pathophysiology of NIDDM.

An increase in β-cell mass is an integral feature of the adaptation to insulin resistance (28,29). The dynamic changes in β-cell mass that occur during the evolution of NIDDM have not been studied in humans because of the inaccessibility of pancreatic tissue. The present studies were therefore undertaken to define the changes in β-cell mass that occur during the progression from insulin resistance with normal glucose tolerance to diabetes in the Zucker diabetic fatty rat. The ZDF represent a failure of β-cell compensation in the face of insulin resistance as compared with Zucker fatty rats, a model in which β-cell compensation is more robust, thereby limiting the extent of the elevation in glucose (30–34).

The data demonstrate that at 5–7 weeks before the onset of diabetes, β-cell mass was similar in the ZDF and ZF rats and was approximately double that of age-matched lean animals. Between this time and 12 weeks, β-cell mass doubled in the ZDF rats as well as Zucker lean rats, while it increased 4-fold in the ZF rats. Thus, at 12 weeks, β-cell mass in the ZDF rats, although twice the value observed in lean animals, represented only 50% of the β-cell mass in the ZF rats. These results provide evidence for a time-dependent failure of β-cell mass to increase sufficiently in the ZDF rat.

Additional experiments explored the mechanisms responsible for these changes and demonstrated that the ZDF rats had a significantly greater average rate of β-cell replication than in the two nondiabetic groups, despite having a significantly lower β-cell mass at 12 weeks than the ZF rats. We concluded, based on the β-cell mass and replication rate meas-

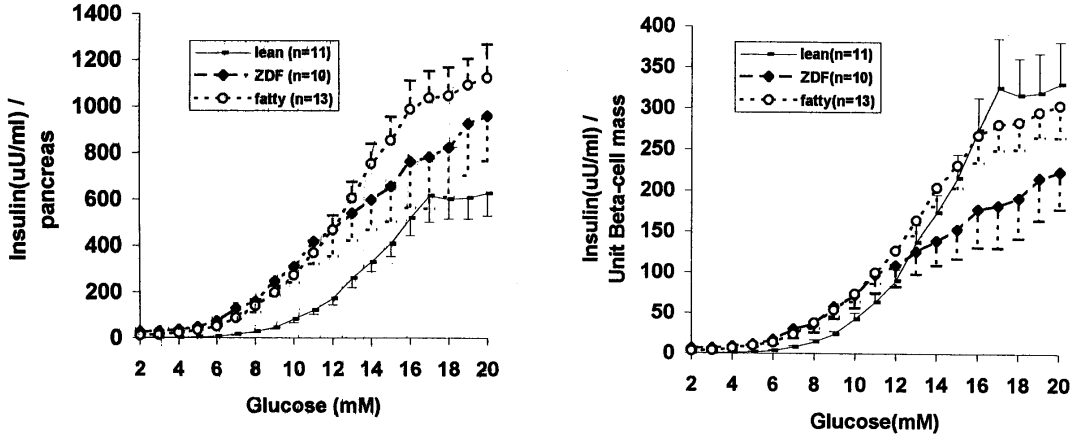

FIG. 3. Insulin secretion rates from 5–7 week perfused male Zucker rat pancreases expressed as insulin secretion (μU/ml) per pancreas (*A*) or as insulin secretion per unit β-cell mass (*B*).

urements, that the measured changes in β-cell mass were due not to a failure of β-cell proliferation (35,36) but to a change in the balance between β-cell neogenesis and death from apoptosis. This change in balance involves a decrease in the rate of neogenesis, an increase in the rate of cell death, or both. Neogenesis (as seen by hormone-positive cells within or budding from the ductal epithelium) was noted in both the ZDF and ZF animals; but as noted above, it is not possible to measure neogenesis rates directly. To analyze these issues further, dynamic changes in β-cell mass were modeled mathematically, and direct morphological evidence of increased cell death by apoptosis in the tissue sections was sought.

Although in ZDF rats from both age-groups, small clusters of lymphocytes are present in the islets and around the ducts, and no histological evidence of inflammation or necrotic cell death was found; therefore, β-cell necrosis can be ruled out. The other form of cell death, apoptosis, is rapid, and its morphological evidence may be present for as little as 1 h (37). Propidium iodide staining showed apoptotic β-cells, albeit in small numbers in the 5- to 7-week-old ZDF rats. Since the calculated decrease of β-cell mass occurs over a 6-week period, it could result from a slow rate of cell death over the whole time period or a faster rate over a shorter time interval between our experimental time points. Our morphological evidence supports the former but cannot rule out the latter. Nonetheless, our data support an increase in the rate of apoptosis rather than a decrease in the rate of neogenesis as the likely major factor responsible for the observed reduction in β-cell mass in diabetic ZDF rats.

These changes in β-cell mass dynamics were associated with progressive morphological abnormalities in ZDF islets with development of irregular-shaped islets and disruption of the mantle of non-β endocrine cells. These disturbances in islet architecture could contribute to the functional defects in insulin secretion that occur with onset of diabetes.

We have previously demonstrated that insulin secretory responses to glucose are retained in prediabetic ZDF rats aged 5–7 weeks (5). However, in those studies, β-cell mass was not measured, and it was not possible to adjust insulin secretory output for changes in β-cell mass. The present studies using the isolated perfused pancreas demonstrate that total pancreatic insulin secretion from the pancreas of ZDF rats is greater than that from the pancreas of lean control rats and similar to that amount secreted by the pancreas of ZF rats. However, when adjusted for changes in β-cell mass, insulin secretory responses appear reduced in the prediabetic ZDF animals, suggesting that even at this stage in the evolution of diabetes, β-cell dysfunction is present, which is masked by increases in β-cell mass (38–40).

Interestingly, at 5–7 weeks the ZDF and ZF rats have similar β-cell mass and body weights, but the ZDF rats have a greater impairment of IPGTT. These findings suggest the presence of β-cell dysfunction or a greater degree of insulin resistance than is present in the ZF rats. As mentioned above, even at 5–7 weeks, insulin secretion is defective in the ZDF animals, whereas the insulin secretory response of the ZF rat approximates that of the ZLC animals. A modest defect in insulin secretion, such as we show, might not be noticed in the heterozygous ZL animals and only becomes unmasked in the face of the increased insulin resistance resulting from mutations in the leptin receptor present in the two fatty groups. With time and exposure to persistent mild hyperglycemia, the function of the β-cells deteriorates further. Here we further identify other differences: inadequate expansion of the β-cell mass in face of insulin resistance, increased β-cell replication rate (perhaps driven by the mild hyperglycemia), and increased estimated β-cell death.

Thus these studies point to a hitherto unappreciated factor, an increase in the rate of β-cell death, as being important in the failure of insulin secretory function. Future work will need to define the factors responsible for the increased rate of cell death and determine whether they are polygenic factors in the genetic background as implied by the studies of Coleman and others (41–44), or whether the increased rate of cell death is due to the presence of increased concentrations of circulating metabolites such as glucose or free fatty acids that limit β-cell survival or other as yet undefined factors that tip the kinetic balance in favor of β-cell death (45–48). Future studies will also need to determine the relative importance of intrinsic defects in the overall function of individual β-cells, versus limitations in β-cell mass in determining the progression to β-cell failure. The answers to these questions will certainly have important implications for understanding the pathophysiology of NIDDM.

In summary, we have shown that the β-cell mass is unable to expand sufficiently to compensate for the insulin resistance and previously documented insulin secretion defects (6,17,34) present in the ZDF rat and that this inadequate response appears to be due to β-cell death exceeding new β-cell formation. This excessive β-cell death most likely results from an increased rate of β-cell apoptosis and raises the importance of defining factors that promote β-cell survival and those that promote β-cell death in models of NIDDM.

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