

Increased β -Cell Apoptosis Prevents Adaptive Increase in β -Cell Mass in Mouse Model of Type 2 Diabetes

Evidence for Role of Islet Amyloid Formation Rather Than Direct Action of Amyloid

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Nondiabetic obese humans adapt to insulin resistance by increasing β -cell mass. In contrast, obese humans with type 2 diabetes have an ~60% deficit in β -cell mass. Recent studies in rodents reveal that β -cell mass is regulated, increasing in response to insulin resistance through increased β -cell supply (islet neogenesis and β -cell replication) and/or decreased β -cell loss (β -cell apoptosis). Prospective studies of islet turnover are not possible in humans. In an attempt to establish the mechanism for the deficit in β -cell mass in type 2 diabetes, we used an obese versus lean murine transgenic model for human islet amyloid polypeptide (IAPP) that develops islet pathology comparable to that in humans with type 2 diabetes. By 40 weeks of age, obese nontransgenic mice did not develop diabetes and adapted to insulin resistance by a 9-fold increase ($P < 0.001$) in β -cell mass accomplished by a 1.7-fold increase in islet neogenesis ($P < 0.05$) and a 5-fold increase in β -cell replication per islet ($P < 0.001$). Obese transgenic mice developed midlife diabetes with islet amyloid and an 80% ($P < 0.001$) deficit in β -cell mass that was due to failure to adaptively increase β -cell mass. The mechanism subserving this failed expansion was a 10-fold increase in β -cell apoptosis ($P < 0.001$). There was no relationship between the extent of islet amyloid or the blood glucose concentration and the frequency of β -cell apoptosis. However, the frequency of β -cell apoptosis was related to the rate of increase of islet amyloid. These prospective studies suggest that the formation of islet amyloid rather than the islet amyloid per se is related to increased β -cell apoptosis in this murine model of type 2 diabetes. This finding is consistent with the hypothesis that soluble IAPP oligomers but not islet amyloid are responsible for increased β -cell apoptosis. The current studies also support the concept that replicating β -cells are more vulnerable to apoptosis, possibly accounting for the failure of β -cell

mass to expand appropriately in response to obesity in type 2 diabetes. *Diabetes* 52:2304–2314, 2003

Inulin resistance is a risk factor for the development of type 2 diabetes (1). The most common cause of insulin resistance is obesity (2). However, most obese humans (~80%) do not develop type 2 diabetes but adapt to chronic insulin resistance by increasing β -cell mass (3–6) and insulin secretion (7). In contrast, the endocrine pancreas in type 2 diabetes is characterized by a deficit in β -cell mass (8,9) and islet amyloid derived from the peptide islet amyloid polypeptide (IAPP) (10,11).

Longitudinal studies in rodents reveal that β -cell mass is adaptively regulated in response to changes in insulin sensitivity. This adaptive change in β -cell mass may be accomplished by changes in the rate of supply and/or loss of β -cells. Supply of β -cells consists of the sum of new islet formation (neogenesis) and β -cell replication within islets, whereas loss consists of β -cell apoptosis (12,13). The predominant supply of β -cells to β -cell mass in humans seems to be islet neogenesis because β -cell replication within islets is rare (3,4). The deficit of β -cell mass in type 2 diabetes seems to be due to an increased frequency of β -cell apoptosis, although these data are based on cross-sectional studies (3).

In humans, measurement of the adaptive changes in β -cell mass or islet turnover over time is unavailable as longitudinal studies of pancreatic morphology cannot be performed. Longitudinal studies in primates with type 2 diabetes report a progressive decline in pancreatic β -cell area with an associated increase in islet amyloid (14–16). However, β -cell mass, islet neogenesis, β -cell apoptosis, and replication were not measured in those studies. A primary goal of the present studies was to establish the time course of changes in β -cell mass and islet turnover leading to a deficit in β -cell mass in an animal model of type 2 diabetes in which pancreatic tissue could be sampled prospectively.

For this purpose, we used obese mice transgenic for human IAPP, a model that develops islet pathology closely resembling that in humans with type 2 diabetes (17). This model allows examination of islet adaptation to obesity without development of diabetes (obese nontransgenic mice versus lean nontransgenic mice), as well as failed

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Received for publication 18 February 2003 and accepted in revised form 21 May 2003.

FFA, free fatty acids; h-IAPP, human islet amyloid polypeptide; IAPP, islet amyloid polypeptide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

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adaptation to obesity with the development of islet amyloid and diabetes (obese transgenic mice versus obese nontransgenic mice). Another advantage of this model is that human IAPP oligomers are a potential mechanism for the increased β -cell apoptosis present in type 2 diabetes (10,11). IAPP is a 37-amino acid peptide coexpressed and cosecreted with insulin by pancreatic β -cells (18). Insulin resistance causes disproportionately increased expression rates of IAPP compared with insulin (19). IAPP has close amino acid homology in the amino and COOH-terminal regions in all species studied (11). In addition, the 20–29 region is homologous in humans, cats, and monkeys and is hydrophobic and amyloidogenic (20,21). In contrast, in mouse IAPP, the 20–29 region has proline substitutions compared with humans, and, as a result, murine IAPP is soluble and nonamyloidogenic (20). Both application of human IAPP to cells and high expression rates of human IAPP by cells induce apoptosis in a concentration-dependent manner, whereas murine IAPP does not have these properties (22–26). The toxic oligomers that induce β -cell apoptosis seem to be small IAPP oligomers that precede the development of IAPP fibrils present in amyloid and act as nonselective ion channels (23,26,27). Mice, unlike cats, humans, and monkeys, do not spontaneously develop type 2 diabetes. However, mice transgenic for human IAPP (h-IAPP) develop diabetes when exposed to conditions that lead to increased expression of IAPP, for example obesity (17,28), high-fat feeding (29), cross-breeding to homozygosity (30), or treatment with growth hormone and glucocorticoids (31).

Therefore, in the present studies, we prospectively measured the β -cell mass, frequency of β -cell replication, islet neogenesis, and β -cell apoptosis in an obese transgenic h-IAPP mouse model from age 10 to 40 weeks to address the following hypotheses. First, obese mice transgenic for h-IAPP have a deficit in β -cell mass. Second, the deficit in β -cell mass is predominantly due to a failed mass expansion (in response to obesity) rather than a decrease in β -cell mass. Third, the mechanism subserving this failure to increase β -cell mass is an increased frequency of β -cell apoptosis that overcomes any increased frequency of β -cell replication and/or islet neogenesis.

RESEARCH DESIGN AND METHODS

Animal model and study design. The mouse model used here has been described previously (17,32). In brief, we have cross-bred a previously developed homozygous h-IAPP transgenic mouse (FVB-*Tg(IAPP)6Jdm/Tg(IAPP)6Jdm*) model (30) with the A^{vy}/a mouse on the C57BL/6 background. The A^{vy}/a mouse possesses a mutation of the agouti gene that causes ubiquitous expression of the encoded 131-amino acid protein (33). The phenotypic consequences of this ectopic expression include the appearance of obesity and related insulin resistance (34). The A^{vy} allele of the agouti locus is dominant to both the A and a alleles and results in the added phenotypic characteristic of a yellow coat color. Therefore, cross-breeding the homozygous h-IAPP mice with agouti yellow mice generates yellow-coated obese mice hemizygous for h-IAPP, referred to as obese TG mice (FVB-*Tg(IAPP)6Jdm/A^{vy}/A*) ($n = 46$ mice), and agouti-coated lean mice hemizygous for h-IAPP, referred to as lean TG mice (FVB-*Tg(IAPP)6Jdm/A/a*) ($n = 28$ mice). We also studied the nontransgenic counterparts of the obese TG and lean TG mice. These were generated in an identical manner except for the substitution of wild-type FVB mice for the FVB-*Tg(IAPP)6Jdm/Tg(IAPP)6Jdm* animals. The resulting yellow-coated obese mice ($n = 46$) are referred to as obese NTG mice, and agouti-coated lean mice ($n = 26$) are referred to as lean NTG mice. It is important to note that all four groups of F1 mice (obese TG, lean TG, obese NTG, and lean NTG) are genetically identical except for two loci: the

transgene insertion site (*Tg(IAPP)6Jdm/+* or *+/+*) and the agouti locus (A^{vy}/A or A/a).

The mice were maintained on a 12-h day/night rhythm with Bantin & Kingman's Standard Rat & Mouse Diet and water ad libitum. Fasted weight and blood glucose values were obtained in the morning after an overnight fast (clean cages and bedding, no food, water ad libitum). Blood glucose values were measured on a tail-tip blood sample with a One Touch II Glucometer (Lifescan, Milpitas, CA). Mice were killed at different ages in the fasted state. A plasma sample for insulin was obtained by cardiac puncture under terminal intraperitoneal pentobarbitone anesthesia, and the pancreases were rapidly dissected from killed animals for morphologic studies (see below).

Insulin assay. Plasma insulin concentration was measured using an in-house competitive colorimetric enzyme-linked immunosorbent assay. Ninety-six-well plates were coated with polyclonal rabbit anti-guinea pig IgG (Dako, Carpinteria, CA) 1:1,000 in coating buffer: 0.1 mol/l Na-bicarbonate (pH 9.8) for at least 16 h at 4°C. After washing, the wells were incubated with guinea pig anti-pig insulin 1:150,000 in incubation buffer: 40 mmol/l phosphate buffer/0.1 mol/l NaCl/0.1% Tween-20 (all Merck)/0.5% human albumin (Behring) for 16 h at 4°C. After another wash, 60 μ l of incubation buffer and 40 μ l of sample, calibrator (rat insulin provided by Novo Nordisk), or quality control together with 25 μ l of peroxidase-labeled insulin (Sigma) 1:50,000 in incubation buffer were left in the wells for 16 h at 4°C. Washing the plates was followed by detecting the peroxidase enzyme activity using 0.46 mmol/l tetra-methyl benzidine dihydrochloride (Sigma) in 0.05 mol/l acetate buffer (pH 5.0) containing 3.2 mmol/l sodium perborate (Merck) for 30 min at room temperature in the dark. The reaction was terminated using 4 mol/l phosphoric acid (Merck). The plates were read at 450 nm using a Dynatech MRX plate reader (Dynex Technologies, Worthing, West Sussex, U.K.). The linear range of this assay was from 30 to 2,000 pmol/l. The mean coefficient of variance for intra-assay duplicates was $6 \pm 1\%$ and for interassay duplicates was $9 \pm 2\%$.

Morphologic techniques. The complete pancreas was rapidly resected from killed mice, all fat and nonpancreas tissue was trimmed, and the pancreas was weighed. The mean weight of the pancreases did not differ between groups at any age. A longitudinal section of the pancreas (tail through head in the flat plane of the pancreas) was fixed in formaldehyde and then embedded in paraffin. Sections of pancreas were then taken through the fixed tissue in the plane of embedding so that a near complete section of pancreas (head, body, and tail) through its maximal width was obtained with each section. These sections were stained for hematoxylin/eosin, insulin, and congo red as described before (29–31). In addition, adjacent sections were immunostained for the marker of replication Ki67 (rat anti-murine Ki67 monoclonal antibody TEC-3, 1/45; Dako) and stained by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method for apoptosis using the TdT-Frag El Kit from Oncogene Research Products (Cambridge, MA) as previously described (3). The β -cell mass for each mouse was measured by first obtaining the fraction of the cross-sectional area of pancreatic tissue (exocrine and endocrine) positive for insulin staining and then multiplying this by the pancreatic weight. In addition, between 30 and 50 islets per mouse were examined in detail as below. For each islet, insulin-positive area (μm^2) and amyloid area (μm^2) were determined by an Olympus IX70 inverted microscope (Olympus America, Melville, NY) connected to a Hewlett Packard computer with Image-Pro plus software (Media Cybernetics, Silver Spring, MD) using the insulin-immunostained and congo red-stained slides. The mean of each of these parameters was computed for each mouse. For the purpose of these studies, insulin-positive cells were considered an islet only when a minimum of five insulin-positive cells were present. While the extent of islet amyloid was computed from the mean islet area positive for congo red (above), the frequency of islet amyloid was analyzed as follows. Cases were subdivided into three groups scored for the frequency of detectable islet amyloid: a score of 1 indicated that $<25\%$ of islets in the sample were positive for amyloid, 2 indicated that 25–75% of islets in the sample were positive for amyloid, and 3 indicated that $>75\%$ of islets were positive for amyloid.

The frequency of β -cell replication for each mouse was calculated by averaging the number of Ki67-positive β -cells in ~ 30 islets from each mouse by comparison of the insulin and Ki67 stains. The frequency of β -cell apoptosis for each mouse was similarly computed by examination of the TUNEL-positive β -cells in the same islets as for the Ki67 stain. The frequency of β -cell replication and β -cell apoptosis was expressed as events per islet. For controlling for different β -cell numbers in different islet cross-sections, the frequency of these events was also computed as the number of positive cells per insulin staining area in each islet. To validate this approach, we needed to confirm that the mean β -cell area was comparable in each group (obese versus lean, and transgenic versus nontransgenic). To this end, we measured the insulin area in five islets from four mice from each of the four groups, two of which were 10 weeks of age and two of which were 40 weeks of age. The number of nuclei were then counted in each of these islets within the insulin

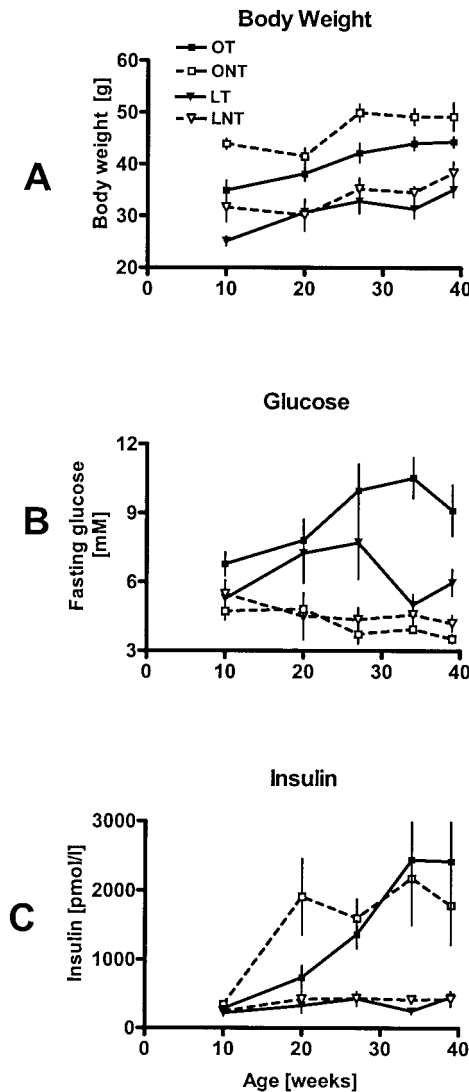


FIG. 1. Body weight (A), fasting blood glucose (B), and fasting plasma insulin (C) in the four groups of mice. OT, obese transgenic; ONT, obese nontransgenic; LT, lean transgenic; LNT, lean nontransgenic.

staining area, and the mean cross-section per β-cell was computed. The results showed no differences between groups, assuring that the use of insulin area is a reasonable surrogate for β-cell number in these studies. To establish a relative frequency of new islet formation (islet neogenesis) between groups, we measured the percentage of exocrine duct cells positive for insulin as previously described (3).

Calculations and statistical analysis. Statistical comparisons were performed using the nonpaired two-tailed Student's *t* test and regression analysis where stated. Data in graphs are presented as means ± SE. Findings were assumed to be statistically significant at the *P* < 0.05 level.

RESULTS

Blood glucose and plasma insulin concentrations

Impact of obesity (obese NTG versus lean NTG mice).

As expected, the nontransgenic agouti viable yellow (obese NT) mice became obese (*P* < 0.001) compared with the nontransgenic controls (lean NT) without the mutation in the agouti gene (Fig. 1). This obesity was present by 10 weeks of age. Neither obese NTG nor lean NTG animals developed diabetes (Fig. 1). The mean fasting insulin concentration was higher (*P* < 0.001) in the obese NTG versus the lean NTG mice, confirming obesity-induced insulin resistance (Fig. 1).

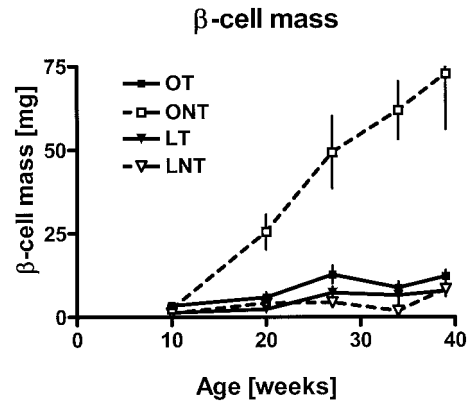


FIG. 2. β-Cell mass with age for the four groups of mice. OT, obese transgenic; ONT, obese nontransgenic; LT, lean transgenic; LNT, lean nontransgenic.

Impact of h-IAPP transgene in obese mice (obese TG versus obese NTG mice). Weight gain (Fig. 1) was attenuated in obese TG versus obese NTG mice, presumably reflecting glycosuria. The fasting blood glucose concentration was already increased in obese TG versus obese NTG mice (6.7 ± 0.5 vs. 4.7 ± 0.4 mmol/l; *P* < 0.05) by 10 weeks of age (Fig. 1). The blood glucose concentration was progressively higher in the obese TG versus obese NTG mice (*P* < 0.001) thereafter. At 20 weeks, the fasting insulin concentration was lower in obese TG versus obese NTG mice, but it was comparable to levels observed in obese NTG mice thereafter (Fig. 1).

Impact of h-IAPP transgene in lean mice (lean TG versus lean NTG mice). Body weight was comparable in the lean TG and lean NTG groups. Blood glucose was also similar at 10 weeks of age, modestly (~7 vs. 5 mmol/l) and transiently increased at age 20–28 weeks in lean TG mice, but comparable again from 30 to 40 weeks of age. The plasma insulin concentrations were comparable in the lean TG and NTG groups throughout the study period.

Islet morphology and β-cell mass

Impact of obesity (obese NTG versus lean NTG mice).

In obese NTG mice, the β-cell mass was already approximately twofold greater than lean nontransgenic mice by 10 weeks of age. This difference increased progressively so that β-cell mass was ninefold greater in obese nontransgenic mice versus lean nontransgenic mice by 40 weeks of age (*P* < 0.001; Figs. 2 and 3). Islet neogenesis assessed by percentage of duct cells positive for insulin was comparable in lean and obese NTG mice until after 20 weeks of age, when it was increased by 1.7-fold (*P* < 0.05) in obese NTG mice (Fig. 4A). The contribution of replication to the increased β-cell mass in obesity is apparent by examining the β-cell replication rate per islet (Fig. 4B) and β-cell replication rate normalized to β-cell area per islet (Fig. 4D). The fractional β-cell replication rate (replication normalized to number of β-cells) was remarkably constant in both lean and obese mice throughout the period of study. However, the actual number of replicating β-cells per islet was progressively higher in obese versus lean mice, as the mean islet insulin area (number of β-cells) was already about twofold greater in obese mice by 10 weeks of age (23,281 ± 7,335 vs. 8,815 ± 3,101 μm²; *P* < 0.05; Table 1). The frequency of β-cell apoptosis was low and no different between obese

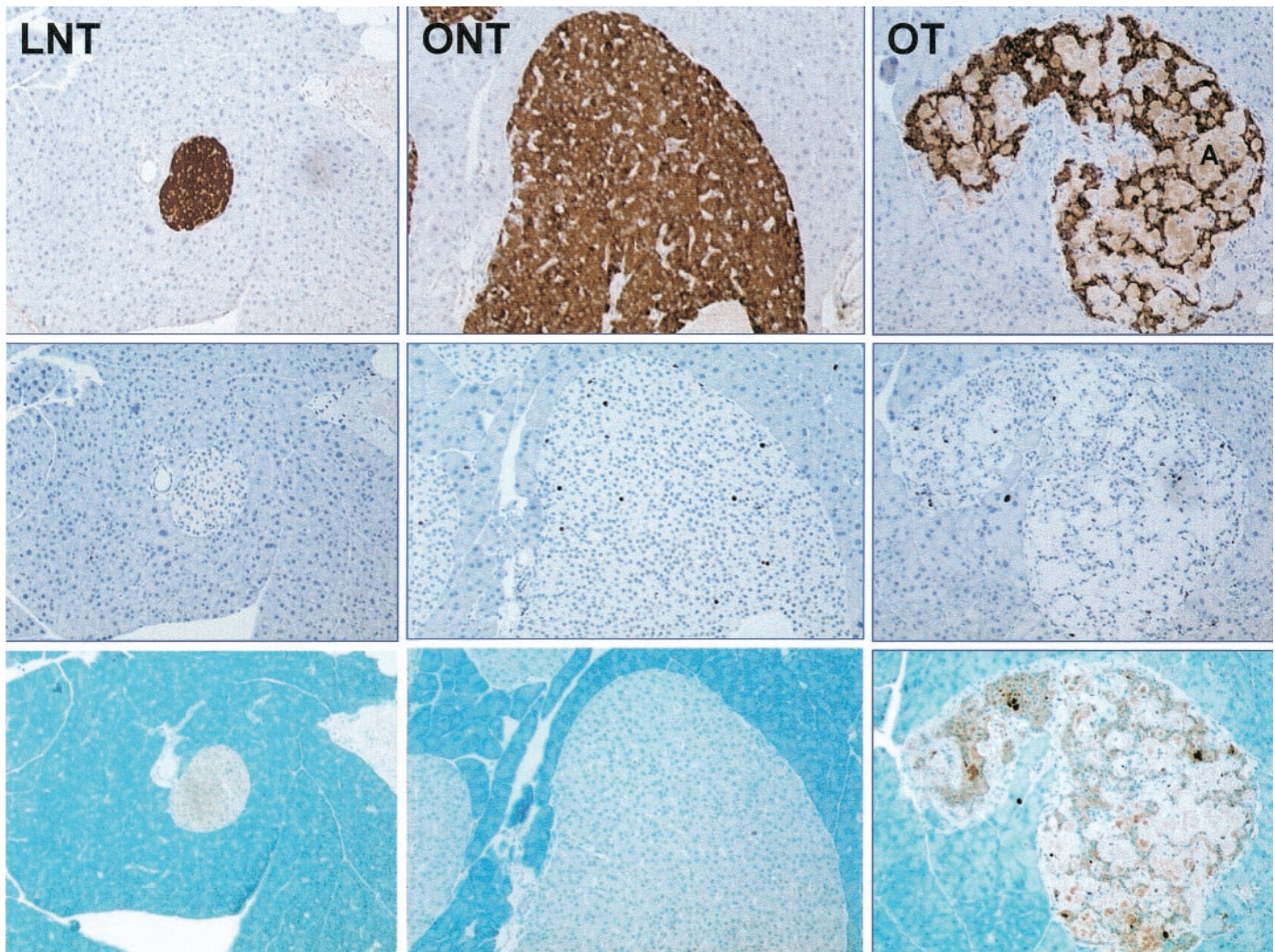


FIG. 3. Islet morphology (sections of mouse pancreas, magnification $\times 10$). The left column shows a typical islet from a lean nontransgenic (LNT) mouse; the central column shows a typical islet from an obese nontransgenic (ONT) mouse; the right column shows a typical islet from an obese transgenic (OT) mouse. The top panels are stained for insulin, the middle panels are stained for replication using the Ki67 marker, and the bottom panels are stained for apoptosis using the TUNEL method. Increased islet size is seen in islets from both OT and ONT mice, with extensive amyloid deposits in the islet from the OT mouse. Numerous replicating cells are present in islets from the obese mice (OT and ONT), although none are present in the islet from the lean mouse (LNT; *middle*). Numerous apoptotic cells are evident in the islet from the OT mouse, as opposed to the islets from nontransgenic mice (LNT, ONT), where apoptosis was a rare event and is not seen in these representative islets (*bottom*).

and lean NTG mice (Figs. 3 and 4). Taken together, these β -cell replication and apoptosis data suggest that the mechanism underlying the increased β -cell mass in the obese mice from 10 to 40 weeks of age was a comparable fractional replication rate in islets that by 10 weeks already had more β -cells. These data suggest that the mechanism for the ongoing increased β -cell mass in the obese mice after 10 weeks of age despite only modest additional weight gain was effective in the fetal and/or the neonatal period to cause increased islet size.

Impact of *h-IAPP* transgene in obese mice (obese TG versus obese NTG mice). Obese TG mice failed to increase β -cell mass in response to obesity compared with obese NTG mice ($P < 0.001$; Fig. 2). The mechanism underlying this failure to increase β -cell mass in response to obesity was a 10-fold increase in the frequency of β -cell apoptosis ($P < 0.001$) in the obese TG versus the obese NTG mice, whereas the frequency of β -cell replication per islet was comparable between these groups (Figs. 3 and 4B), although the frequency of β -cell replication per islet

when normalized to β -cell area was increased in both lean and obese transgenic mice (Fig. 4D). Islet neogenesis, although $\sim 50\%$ increased ($P < 0.001$) in obese TG versus obese NTG mice, presumably could not compensate for the 10-fold increase in β -cell apoptosis (Figs. 3 and 4). Inspection of the TUNEL-stained islets showed that the TUNEL-positive cells appeared frequently in pairs (Fig. 5A), consistent with postmitotic apoptosis, suggesting that replicating cells may have increased vulnerability to apoptosis. This impression is supported by the close relationship between the frequency of replication and apoptosis (Fig. 5B). **Impact of *h-IAPP* transgene in lean mice (lean TG versus lean NTG mice).** β -Cell mass was comparable in lean TG and NTG mice (Fig. 2), despite a fivefold ($P < 0.001$) increase in the frequency of β -cell apoptosis in lean TG mice (Fig. 6). The latter was presumably offset by the twofold ($P < 0.001$) increased neogenesis and threefold ($P < 0.001$) increased β -cell replication per islet in lean TG versus NTG mice (Fig. 6).

TABLE 1
Islet morphology at age 10 and 40 weeks

Age (weeks)	LNT	ONT	LT	OT
β-Cell mass (mg)				
10	1.14 ± 0.56	2.39 ± 0.77	1.35 ± 0.23	3.42 ± 0.77
40	8.37 ± 2.24	72.78 ± 16.64	7.87 ± 1.77	12.21 ± 1.89*
Insulin area/islet (μm ²)				
10	8,815 ± 3,101	23,281 ± 7,335	4,107 ± 796#	8,747 ± 1,478*
40	21,043 ± 3,686	100,612 ± 25,928†	13,139 ± 2,670	14,806 ± 1,850*
Replication (cells/islet)				
10	0.59 ± 0.15	0.25 ± 0.04	0.96 ± 0.17	3.42 ± 0.61*
40	0.48 ± 0.08	3.03 ± 0.89	1.77 ± 0.72	4.64 ± 0.66
Replication (cells/insulin area μm ²)				
10	1.1 × 10 ⁻⁴ ± 0.3 × 10 ⁻⁴	1.7 × 10 ⁻⁵ ± 0.6 × 10 ⁻⁵ †	3.8 × 10 ⁻⁴ ± 1.8 × 10 ⁻⁴ ‡	4.5 × 10 ⁻⁴ ± 1.0 × 10 ⁻⁴ *
40	2.4 × 10 ⁻⁵ ± 0.4 × 10 ⁻⁵	3.5 × 10 ⁻⁵ ± 1.3 × 10 ⁻⁵	2.5 × 10 ⁻⁴ ± 1.7 × 10 ⁻⁴ ‡	3.8 × 10 ⁻⁴ ± 0.8 × 10 ⁻⁴ *
Apoptosis (cells/islet)				
10	0.08 ± 0.03	0.02 ± 0.02	0.19 ± 0.04#	1.23 ± 0.35§
40	0.03 ± 0.01	0.11 ± 0.05	0.26 ± 0.09#	0.42 ± 0.04*
Apoptosis (cells/insulin area μm ²)				
10	2.1 × 10 ⁻⁵ ± 1.2 × 10 ⁻⁵	8 × 10 ⁻⁷ ± 7 × 10 ⁻⁷ †	8.2 × 10 ⁻⁵ ± 3.6 × 10 ⁻⁵ #	1.3 × 10 ⁻⁴ ± 0.2 × 10 ⁻⁴ *
40	1.8 × 10 ⁻⁶ ± 0.9 × 10 ⁻⁶	1.2 × 10 ⁻⁶ ± 0.6 × 10 ⁻⁶	3.3 × 10 ⁻⁵ ± 1.8 × 10 ⁻⁵ ‡	3.5 × 10 ⁻⁵ ± 0.7 × 10 ⁻⁵ *
New islet formation (% insulin-positive duct cells)				
10	0.23 ± 0.02	0.30 ± 0.07	0.58 ± 0.12#	0.72 ± 0.06*
40	0.31 ± 0.09	0.50 ± 0.19	0.50 ± 0.13	0.82 ± 0.23

LNT, lean nontransgenic; ONT, obese nontransgenic; LT, lean transgenic; OT, obese transgenic. *ONT vs. OT, *P* < 0.001; †LNT vs. ONT, *P* < 0.001; ‡LNT vs. LT, *P* < 0.001; §ONT vs. OT, *P* < 0.05; ||LNT vs. ONT, *P* < 0.05; #LNT vs. LT, *P* < 0.05.

Relationship among β-cell apoptosis, blood glucose, and islet amyloid. Obese TG mice developed large extracellular deposits of islet amyloid comparable to those seen in humans with type 2 diabetes (Fig. 3). These deposits were first evident by ~10 weeks of age (small occasional deposits present in five of eight 10-week-old mice) and progressively increased in size and extent in obese TG mice until 30 weeks of age, when these indexes reached a plateau (Fig. 7). Islet amyloid deposits were present in only a minority of lean TG mice (~5%), but when they did occur, they were much less frequent and extensive than in the obese TG mice (Fig. 7). No islet amyloid was present in NTG mice.

To address the potential relationship between the extracellular islet amyloid and β-cell apoptosis, we examined the time course of these parameters in lean and obese TG mice versus the frequency of β-cell apoptosis (Fig. 8). There was no relationship between the extent (or frequency) of islet amyloid and the frequency of β-cell apoptosis (*r* = 0.06, *P* = 0.59; Fig. 8). Indeed, inspection of the time course of β-cell apoptosis (Fig. 4) showed a maximal frequency of β-cell apoptosis when islet amyloid (Fig. 7) was minimal (10 weeks of age), with a frequency of β-cell apoptosis decreasing at a time that islet amyloid was maximal (40 weeks). However, when the frequency of β-cell apoptosis was compared with the increment in islet amyloid (compared with the previous sampling time point), a relationship was evident (*r* = 0.77, *P* < 0.001; Fig. 8). These data suggest that formation of islet amyloid rather than islet amyloid per se is related to increased β-cell apoptosis.

Finally, we found no relationship between the frequency of β-cell apoptosis and the blood glucose concentration (*r* = 0.05, *P* = 0.7; Fig. 8). Furthermore, the 17 of 46 obese transgenic mice with a blood glucose of <4.5 mmol/l had a higher frequency of β-cell apoptosis (17-fold greater than

nontransgenic controls) than the 11-fold increment in β-cell apoptosis present in the obese transgenic mice as a whole. We conclude that hyperglycemia per se does not seem to be a primary factor in increased β-cell apoptosis in this model (Fig. 8).

DISCUSSION

In the present studies, we report that nontransgenic mice adapted to obesity with a ninefold increase in β-cell mass, which was accomplished by a twofold increase in new islet formation and a fivefold increase in β-cell replication per islet. The latter was due to a constant fractional β-cell replication rate in progressively larger islets. The islets in obese nontransgenic mice already had an increased β-cell area by 10 weeks of age. In contrast, mice that were both obese and transgenic for h-IAPP failed to increase β-cell mass, with a resulting marked deficit (~80%) in β-cell mass compared with obese nontransgenic mice. The underlying mechanism subserving this failure to expand β-cell mass was a 10-fold increased frequency of β-cell apoptosis.

It is not possible to perform longitudinal studies of pancreatic morphology in humans. We therefore resorted to a murine model of type 2 diabetes to gain insights into the mechanisms of the reported deficit in β-cell mass in humans with type 2 diabetes. Specifically, we questioned whether this deficit is due to a loss of β-cell mass or a failure of β-cell mass to expand appropriately. Also, we questioned whether the mechanism subserving this deficit is insufficient islet neogenesis and/or β-cell replication or an increased frequency of β-cell apoptosis. Use of any animal model has the limitation that it is not certain to reproduce human pathophysiology. However, the murine model used in the current studies has the advantage of resembling humans with type 2 diabetes in several ways.

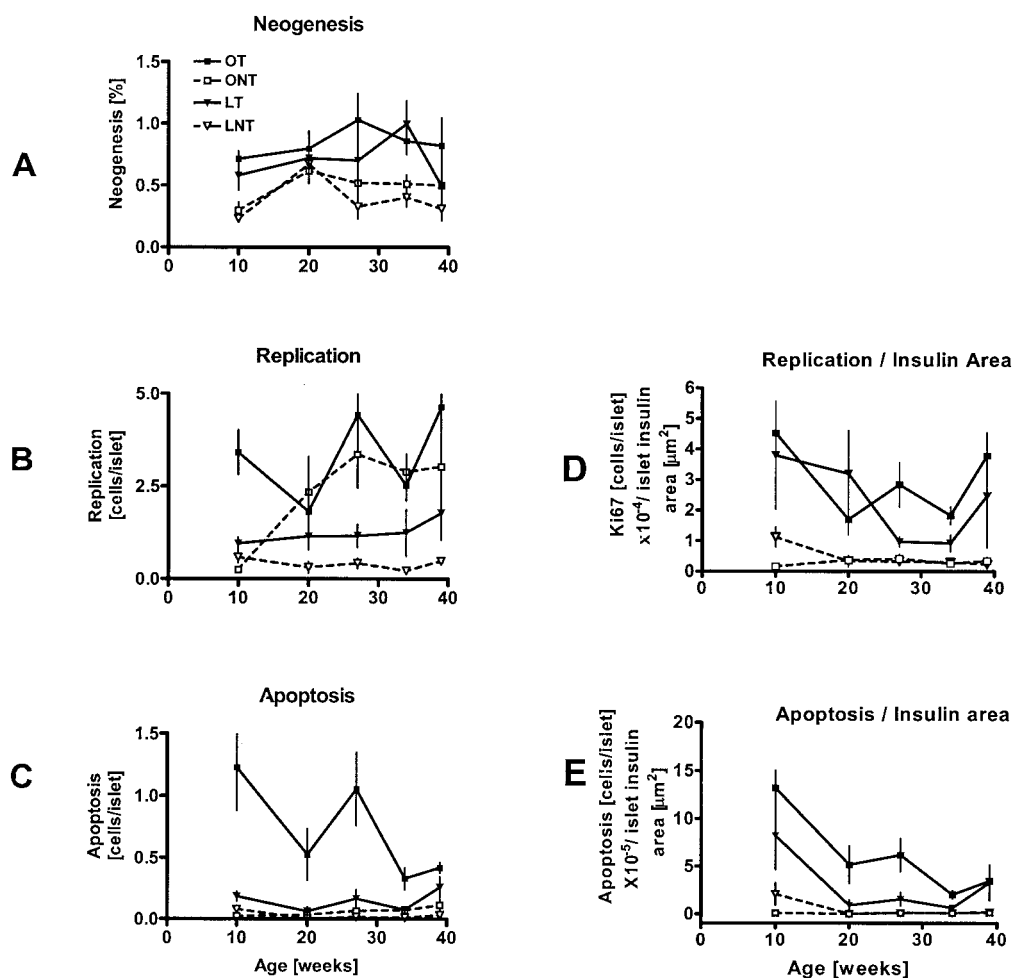


FIG. 4. New islet formation (A), β -cell replication/islet (B), β -cell apoptosis/islet (C), β -cell replication/insulin area (D), and apoptosis/insulin area (E) with age in the four mice groups. OT, obese transgenic; ONT, obese nontransgenic; LT, lean transgenic; LNT, lean nontransgenic.

Obese transgenic mice are more prone to develop diabetes than lean transgenic mice. Also, obese transgenic mice have a clinical time course comparable to humans with type 2 diabetes, with disease onset in midlife characterized by progressive hyperglycemia and impaired insulin secretion (17). Most important, given the focus of these studies, obese transgenic mice develop islet pathology resembling that in humans with type 2 diabetes with islet amyloid, a comparable deficit in β -cell mass ($\sim 80\%$ vs. $\sim 65\%$) and a comparable increased frequency of β -cell apoptosis (9-fold vs. 3- to 10-fold) (3,6,8). We therefore focused on the time course changes in islet mass and turnover in the current murine model in an attempt to gain insight into the pathophysiology of the β -cell deficit reported in cross-sectional studies of humans with type 2 diabetes.

These time course studies revealed that the deficit in β -cell mass developed predominantly as a result of a failed increase in β -cell mass (in response to the insulin resistance of obesity) rather than a loss of β -cell mass. This model of a failed adaptive increase in β -cell mass to insulin resistance is compatible with epidemiology data in humans. In humans who are genetically predisposed to type 2 diabetes, onset of hyperglycemia often occurs during pregnancy (35), a period of insulin resistance when β -cell mass adaptively increases in rodents (36,37). Likewise, diabetes onset may occur after induction of insulin resis-

tance by use of glucocorticoid treatment (38). Age of onset of type 2 diabetes tends to track age of onset of obesity, which both are now common in childhood (39). Furthermore, diabetes onset is now commonly encountered in obese teenagers in relation to puberty, a circumstance whereby the insulin resistance of obesity and puberty are superimposed (40). Each of these outcomes could be explained by a failure to adaptively increase β -cell mass in response to insulin resistance.

The deficit in β -cell mass in obese transgenic mice was due to increased β -cell apoptosis. Islet neogenesis and β -cell replication per islet both increased comparably in obese transgenic and obese nontransgenic mice. These data are also comparable to the cross-sectional data available in human studies, in which islet neogenesis was increased comparably in obese humans with and without type 2 diabetes while the frequency of β -cell apoptosis was increased in type 2 diabetes (3). β -Cell replication is rare in humans and not different between humans with and without type 2 diabetes (3,4). Therefore, the current obese transgenic mouse model and humans with type 2 diabetes share a deficit in β -cell mass as a result of increased β -cell apoptosis that exceeds adaptive increased rates of β -cell supply. In the current murine model, we are able to report that this increased frequency of β -cell apoptosis precedes the development of diabetes and is sustained throughout

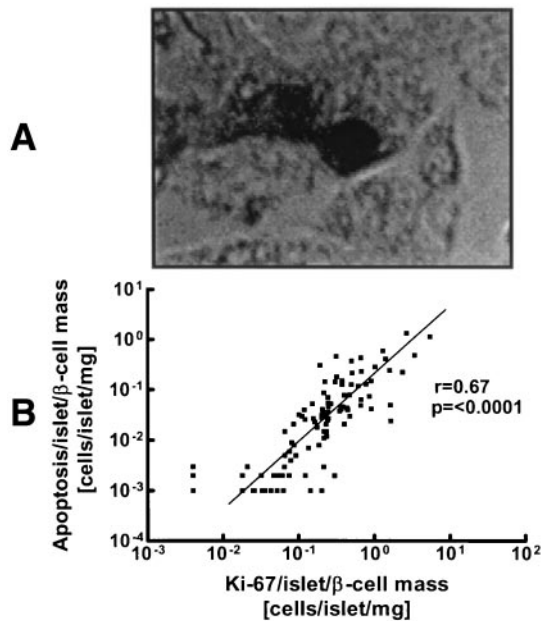


FIG. 5. A: Pairs of apoptotic β-cells in islet tissue of transgenic mice as evidence of postmitotic apoptosis (magnification ×100). **B:** Correlation of apoptosis per islet per β-cell mass with Ki67 per islet per β-cell mass for all groups of mice.

the time course of the studies. As previously reported in another rodent model (41) and in *in vitro* studies (42), we also observed a pattern of pairs of apoptotic β-cells, consistent with an increased predisposition of replicating β-cells to apoptosis. If replicating β-cells in the obese transgenic mice are those that have an increased predisposition to apoptosis, then the anticipated consequence would be a failure to expand β-cell mass rather than a decrease in β-cell mass.

The lean transgenic mice also provide some interesting insights. Although they do not develop diabetes and have a comparable β-cell mass to lean nontransgenic mice, they do have an increased frequency of β-cell apoptosis compared with the lean nontransgenic mice. However, the 5-fold increased frequency of β-cell apoptosis was half the 10-fold increased frequency of apoptosis observed in obese transgenic mice. This finding suggests that there might be a threshold rate of β-cell apoptosis beyond which islet neogenesis and β-cell replication are unable to sustain β-cell mass. This threshold is presumably defined by the sum of the maximal potential rates of neogenesis and β-cell replication. Rodents have a much higher capacity for β-cell replication than humans but seem to be capable of a similar increment in islet neogenesis as humans. It therefore seems reasonable to postulate that rodents have a greater capacity to sustain increased rates of β-cell apoptosis than humans before diabetes supervenes. Also, because there does seem to be a decreased capacity for islet neogenesis and β-cell replication with aging in humans (3), one might anticipate that the ability to compensate for increased β-cell apoptosis with age also declines. Both islet neogenesis and β-cell replication were increased in the lean transgenic mice versus the lean nontransgenic mice in the present study, presumably as a consequence of the subtle rise in blood glucose (~7 vs. 5 mmol/l) in these mice, because they did not seem to be

insulin resistant compared with lean nontransgenic mice. If the deficit in β-cell mass in humans with type 2 diabetes is due to an increased frequency of β-cell apoptosis, then efforts to prevent the development of type 2 diabetes or reverse the loss of β-cell mass in established type 2 diabetes should focus on inhibition of the accelerated β-cell apoptosis (3,6,8). Efforts in this regard require an understanding of the mechanisms that lead to increased β-cell apoptosis.

A number of potential mechanisms for increased β-cell apoptosis in type 2 diabetes have been postulated. These include hyperglycemia (43,44) and increased free fatty acid (FFA) concentrations (45,46). Although it is clear that high glucose concentrations per se can induce β-cell apoptosis, in the present mouse model, the frequency of apoptosis increased before the onset of hyperglycemia. Once hyperglycemia is present, it may have contributed to increased β-cell apoptosis, although we were unable to find a relationship between the frequency of apoptosis and the blood glucose concentration (Fig. 8). However, it should be emphasized that the current studies were not designed to address the relationship between glycemia and β-cell apoptosis and certainly do not rule out an important contribution of hyperglycemia on increased β-cell apoptosis, particularly in humans. In the present studies, the plasma FFA concentrations were comparable in the obese transgenic and obese nontransgenic mice (data not shown). However, the FFA concentration exposed to the islet will depend on the actions of lipoprotein lipase on circulating triglycerides rather than the plasma FFA. The islet FFA exposure is therefore unknown *in vivo*. Another potential explanation for increased β-cell apoptosis in type 2 diabetes is the actions of IAPP oligomers (14–16).

The current murine model transgenic for h-IAPP provides an opportunity to examine in greater detail the potential role of IAPP on the increased frequency of β-cell apoptosis in type 2 diabetes. The role of IAPP amyloid in the development of type 2 diabetes remains controversial. Although it has long been recognized that islet amyloid derived from IAPP is a characteristic of type 2 diabetes (47–50), it has been debated whether islet amyloid is secondary to or a cause of the disease. Several previous observations contradict a mechanistic role of islet amyloid in the pathogenesis of islet dysfunction in type 2 diabetes. Not all islets in patients with type 2 diabetes have islet amyloid (3,49,50). Although humans with impaired fasting glucose have an ~40% deficit in β-cell mass, they have no more islet amyloid than nondiabetic individuals (3). The present longitudinal studies allow us to examine the relationship between the development of islet amyloid and the mechanism underlying the deficit in β-cell mass, *i.e.*, increased apoptosis. If we examine these parameters only at 40 weeks (Fig. 9) in a cross-sectional manner, then we might conclude that islet amyloid causes increased β-cell apoptosis, loss of β-cell mass, and hyperglycemia. However, when we examine the time course of these parameters, it becomes clear that there is not a positive relationship between the increased frequency of β-cell apoptosis and the extent of islet amyloid (Figs. 7 and 8). In fact, if anything, as islet amyloid reaches its plateau in obese TG mice, there is a suggestion of some decrease in β-cell apo-

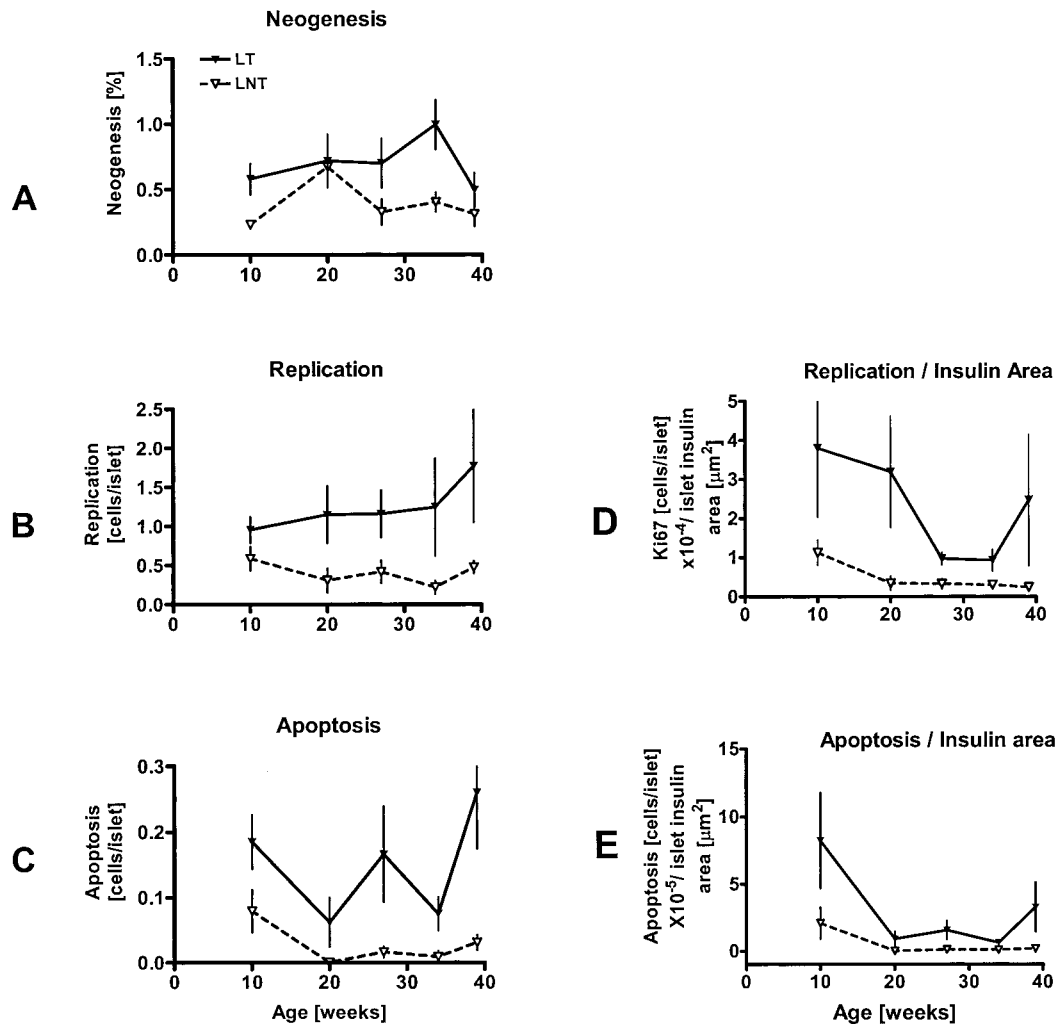


FIG. 6. New islet formation (A), β -cell replication/islet (B), β -cell apoptosis/islet (C), β -cell replication/insulin area (D), and β -cell apoptosis/insulin area (E) with age in the lean groups only. LT, lean transgenic; LNT, lean nontransgenic.

ptosis. However, we do find a positive relationship between the increment in islet amyloid in the previous sampling interval and the frequency of β -cell apoptosis. This finding supports the hypothesis that formation of islet amyloid rather than islet amyloid per se is related to the increased frequency of β -cell apoptosis in transgenic mice. It is important to stress here that amyloid is defined by the large deposits of protein present in the form of nonbranching amyloid fibrils, most conveniently detected and quantified by congo red or thioflavine T staining. There has also been some controversy as to whether h-IAPP-derived amyloid is toxic. In type 2 diabetes, β -cells are commonly adjacent to islet amyloid deposits and yet seem to be viable. Indeed, this is evident in the current murine model (Fig. 3). Furthermore, we previously reported that homozygous transgenic mice for h-IAPP (with high expression rates of h-IAPP) developed diabetes and lost β -cell mass before development of islet amyloid (30), an observation consistent with the present study in which the increased frequency of β -cell apoptosis precedes the development of islet amyloid in most islets. Although each of these observations contradicts a role of islet amyloid deposits visible by microscopy in the loss of β -cell mass in type 2 diabetes, they do not preclude a role of IAPP oligomers too small to be visible by light microscopy.

There is increasing evidence that such IAPP oligomers act as nonselective ion channels in cell membranes to induce apoptosis (16,26,27). We propose that the toxic oligomers of h-IAPP are formed from intermediates that might develop into either amyloid fibrils or toxic soluble oligomers (Fig. 10). As amyloid (defined by congo red staining extracellular deposits) is insoluble, it will progressively accumulate as observed in the present studies. Our longitudinal studies allow us to affirm the *in vitro* findings that these amyloid deposits do not directly cause β -cell apoptosis (23,42). In contrast, our observation that the rate of islet amyloid formation and therefore presumably the rate of formation of toxic oligomers was related to β -cell apoptosis is consistent with recent studies that have led to the model proposed in Fig. 10. The alternative pathways for formation of toxic oligomers and islet amyloid fibrils from a common intermediate postulated in Fig. 10 could also account for the fact that the formation of these toxic oligomers and islet amyloid fibrils may progress at different rates under different conditions. Most recently, it has been reported that the structure of soluble toxic oligomers composed of the three different amyloidogenic proteins IAPP, synuclein, and Alzheimer's β protein have such a common structure that they can be identified by the same antibody (51). This antibody does not detect

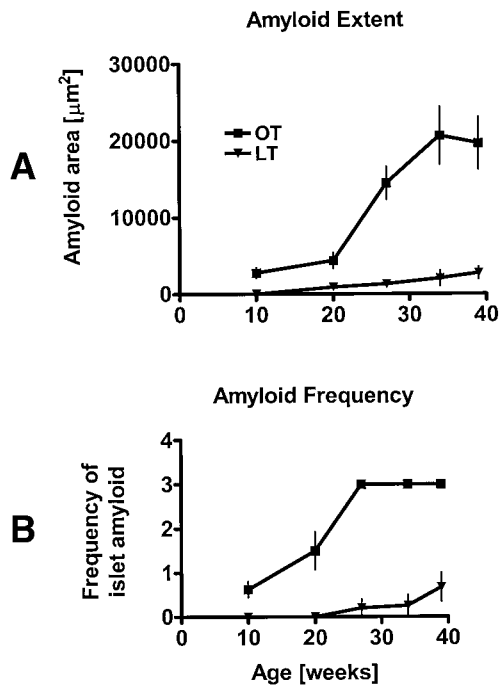


FIG. 7. Extent (A) and frequency (B) of islet amyloid (OT and LT mice).

either the monomers or the amyloid forms of these proteins. The antibody revealed that the distribution of soluble toxic oligomers of Alzheimer's β protein is distinct from amyloid and was more highly correlated with presence of dementia than amyloid. These findings support the proposed model in Fig. 10. If the model proposed in Fig. 10 is accurate, then important questions that still remain are where and why do toxic oligomers of IAPP form?

It is unresolved whether IAPP oligomers form and act predominantly intracellularly (25,30,52), disrupting membranes such as endoplasmic reticulum and Golgi, and/or extracellularly (14–16). H-IAPP expression increases with insulin resistance (19). One possibility is that once h-IAPP expression passes a threshold, the cells are no longer able to chaperone this protein and the resulting free h-IAPP monomers begin to oligomerize in the aqueous environment of the cell as they do in vitro (53,54). This model is consistent with transgenic mice studies. On an FVB background, transgenic mice hemizygous for h-IAPP do not develop diabetes or islet amyloid, but when the gene dosage is doubled by cross-breeding to homozygosity, the mice develop diabetes characterized by β-cell loss and intracellular h-IAPP oligomerization (30). Induction of insulin resistance and increased h-IAPP expression by pharmacologic means (growth hormone and dexamethasone) (31) by a high-fat diet (29) or obesity (10) also lead to diabetes in hemizygous transgenic mice (30). Although this concept of a maximal threshold for h-IAPP trafficking may explain the development of type 2 diabetes in h-IAPP transgenic mice, it does not explain why most obese humans tolerate insulin resistance with increased insulin secretion from an enlarged β-cell mass, whereas those genetically predisposed to type 2 diabetes have an ~70% loss in β-cell mass, have increased β-cell apoptosis, and develop islet amyloid (3). We have hypothesized in the past that the last may be due to inherited polymorphisms

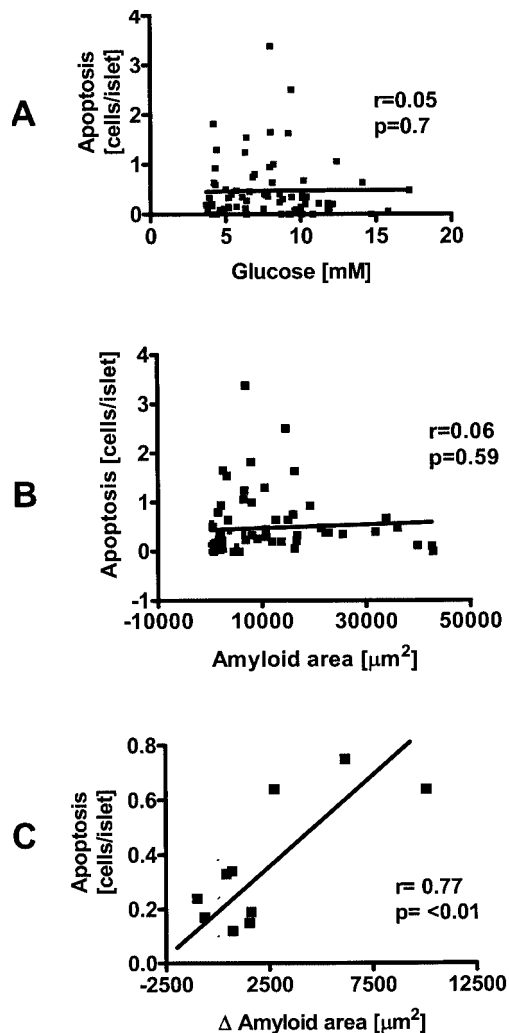


FIG. 8. A: Lack of correlation between the frequency of β-cell apoptosis and fasting blood glucose in transgenic mice. B: Lack of correlation between the frequency of β-cell apoptosis/islet and amyloid area/islet in transgenic mice. C: Correlation between frequency of β-cell apoptosis/islet and increment in islet amyloid/islet (since previous pancreas sampling). Group means for each sampling interval ($n = 5$) in lean and obese transgenic mice are shown ($n = 10$ total).

in one or more chaperone proteins that results in a decreased capacity for h-IAPP binding, although there is no evidence to support this as yet (53). Alternatively, the development of islet amyloid and any adverse effects caused by it may be secondary to hyperglycemia in type 2 diabetes and unrelated to the underlying genetic predisposition.

In summary, in the present studies, obese mice transgenic for h-IAPP failed to appropriately expand β-cell mass in response to obesity as a consequence of increased β-cell apoptosis. Although no prospective study of β-cell dynamics can be undertaken in humans, it is intriguing to pose the possibility that a similar phenomenon may be present in humans with type 2 diabetes. This model is consistent with cross-sectional studies in humans with type 2 diabetes at death, revealing a deficit in β-cell mass relative to weight-matched controls and increased β-cell apoptosis. From these prospective studies, we conclude that islet amyloid does not directly cause the increased frequency of β-cell apoptosis and therefore the β-cell deficit in this model. Instead, these data suggest that the process of islet amyloid formation from IAPP monomers,

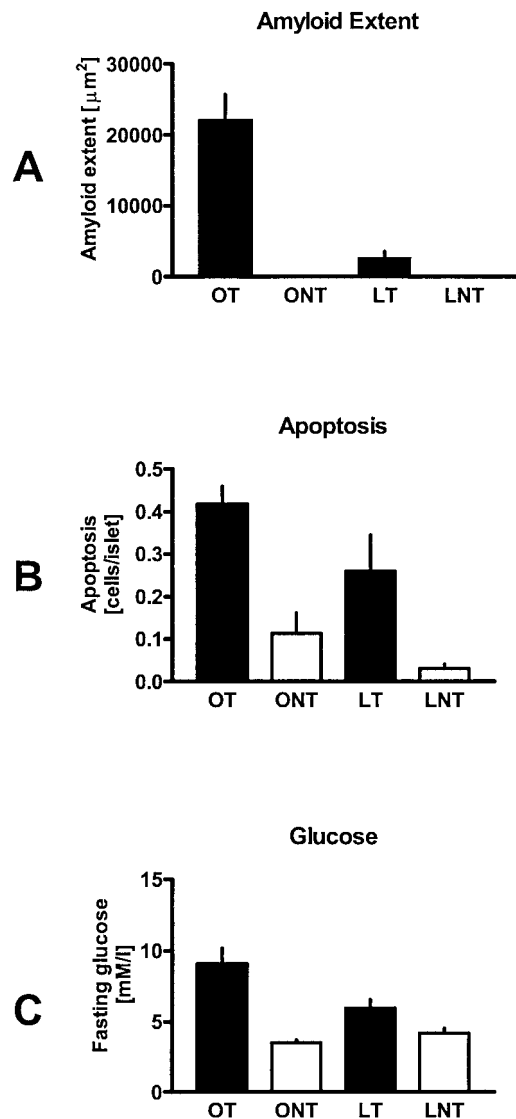


FIG. 9. Amyloid extent (A), frequency of apoptosis (B), and fasting glucose (C) in the four groups of mice at the 40-week time point. If this had been the only sampling interval, then it might seem that the amyloid directly caused the increased apoptosis in contrast to the findings in this longitudinal study (see Fig. 8).

presumably through intermediate-sized IAPP oligomers, is related to the frequency of β -cell apoptosis. Future studies are required to identify the structure of these oligomers, why they form in people who are predisposed to type 2 diabetes, and the mechanism by which they induce β -cell apoptosis.

ACKNOWLEDGMENTS

These studies were funded by a project grant from the Wellcome Trust (004937) and a National Institutes of Health grant (DK61539).

We appreciate the constructive suggestions of our colleagues Robert Ritzel, Robert Chow, Leena Haataja, Chia-Yu Lin, and Darren Michael and our collaborator Charles Glabe at University of California Irvine. We acknowledge the excellent technical assistance of Susan McIntyre, Fiona Gulliver-Sloan, Joan Yorke, and Jaqi Armstrong (University of Edinburgh), and Lucretia Howard and Tatyana Gurlo (University of Southern California).

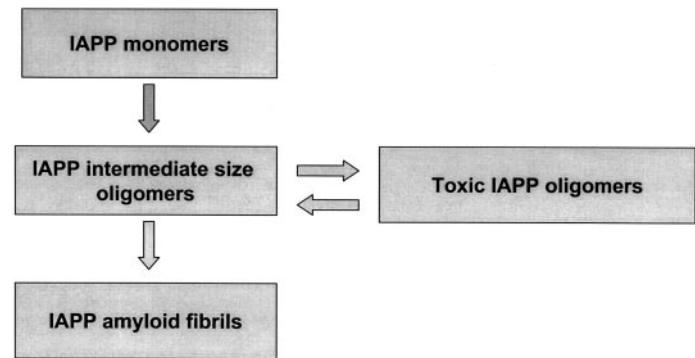


FIG. 10. An emerging model for the relationship among protein monomers, amyloid formation, and the toxic form of amyloidogenic protein oligomers (23,27). Amyloid is defined by protein deposits identified by congo red or thioflavine T staining and is formed from monomers via intermediate forms. The amyloid deposits in both in vitro and the current studies are not directly toxic and, as they are insoluble, simply accumulate with time. Toxic oligomers can be formed from amyloidogenic monomers, which share close structural features even when derived from distinct protein monomers (51). The rate of formation of amyloid in the current study may provide insight into the quantity of toxic oligomers (see Fig. 8), whereas the quantity of amyloid at any time presumably simply reflects the accumulation of amyloid over time.

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