

Spontaneous Diabetes in Hemizygous Human Amylin Transgenic Mice That Developed Neither Islet Amyloid nor Peripheral Insulin Resistance

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OBJECTIVES—We sought to 1) Determine whether soluble-misfolded amylin or insoluble-fibrillar amylin may cause or result from diabetes in human amylin transgenic mice and 2) determine the role, if any, that insulin resistance might play in these processes.

RESEARCH DESIGN AND METHODS—We characterized the phenotypes of independent transgenic mouse lines that display pancreas-specific expression of human amylin or a nonaggregating homolog, [^{25,28,29}Pro]human amylin, in an FVB/n background.

RESULTS—Diabetes occurred in hemizygous human amylin transgenic mice from 6 weeks after birth. Glucose tolerance was impaired during the mid- and end-diabetic phases, in which progressive β -cell loss paralleled decreasing pancreatic and plasma insulin and amylin. Peripheral insulin resistance was absent because glucose uptake rates were equivalent in isolated soleus muscles from transgenic and control animals. Even in advanced diabetes, islets lacked amyloid deposits. In islets from nontransgenic mice, glucagon and somatostatin cells were present mainly at the periphery and insulin cells were mainly in the core; in contrast, all three cell types were distributed throughout the islet in transgenic animals. [^{25,28,29}Pro]human amylin transgenic mice developed neither β -cell degeneration nor glucose intolerance.

CONCLUSIONS—Overexpression of fibrillogenic human amylin in these human amylin transgenic mice caused β -cell degeneration and diabetes through mechanisms independent from both peripheral insulin resistance and islet amyloid. These findings are consistent with β -cell death evoked by misfolded but soluble cytotoxic species, such as those formed by human amylin *in vitro*. *Diabetes* 57:2737–2744, 2008

Increasing evidence indicates that decreased β -cell mass contributes to the impaired insulin secretion characteristic of type 2 diabetes (1–3). Amylin, also referred to as islet amyloid polypeptide, is a 37-amino acid polypeptide (4,5) secreted from pancreatic islet β -cells whose aggregation results in islet amyloid formation in type 2 diabetes (6). Islet amyloid has been

reported in 40–90% of pancreases from type 2 diabetic subjects studied post mortem (7–11) and has been linked to both decreased β -cell mass and β -cell dysfunction (12,13). *In vitro*, human amylin causes apoptosis of islet β -cells, and there is growing evidence that this pathogenic process may contribute to the β -cell deficit in type 2 diabetes (1,2,14,15). However, it remains unresolved whether islet amyloid contributes to the etiopathogenesis of type 2 diabetes or, by contrast, occurs only as a consequence of the disease.

Several independent lines of human amylin transgenic mice have been developed to investigate the role of amylin and islet amyloid in the pathogenesis of type 2 diabetes (16–19). The findings and conclusions from phenotypic characterization studies are wide ranging and sometimes at variance. Transgenic animals developed by several research groups did not develop spontaneous diabetes or insulin resistance or exhibit evidence of islet amyloid formation, suggesting that overexpression of human amylin alone was not sufficient to contribute to diabetes development and islet amyloid formation in those models (16–18). In contrast, Janson et al. (19) showed development of spontaneous diabetes in the absence of islet amyloid in homozygous individuals from a further transgenic mouse model, consistent with the view that overexpression of human amylin is sufficient for diabetes development but not islet amyloid formation in that model. It was previously thought that overexpression of human amylin might be sufficient for islet amyloid formation, but some studies have suggested that insulin resistance might also be necessary (20–22).

Evidence concerning the role of human amylin in the processes that lead to or cause diabetes remains conflicting, and a clear role for human amylin-mediated β -cell death has not been established at this time, at least in part due to conflicting evidence from the different lines of human amylin transgenic mice. Previous reports have described the noticeable lack of correlation between amyloid deposition and hyperglycemia in other transgenic models of amylin-induced diabetes (21,23). Islets from homozygous individuals from the FVB/n-based line reported by Janson et al. (19) demonstrated a pattern of β -cell loss that closely reflects that in islets from human type 2 diabetic patients (1,3,9), but hemizygous animals from that line reportedly do not develop diabetes.

Here, we report a transgenic human amylin mouse model (L13) in which hemizygous individuals developed early-onset diabetes without peripheral insulin resistance and islet amyloid formation. We demonstrate that the disappearance of functional β -cells during the progression of diabetes in this model contributes to the pathogenesis of diabetes. The absence of islet amyloid in the pancreas of

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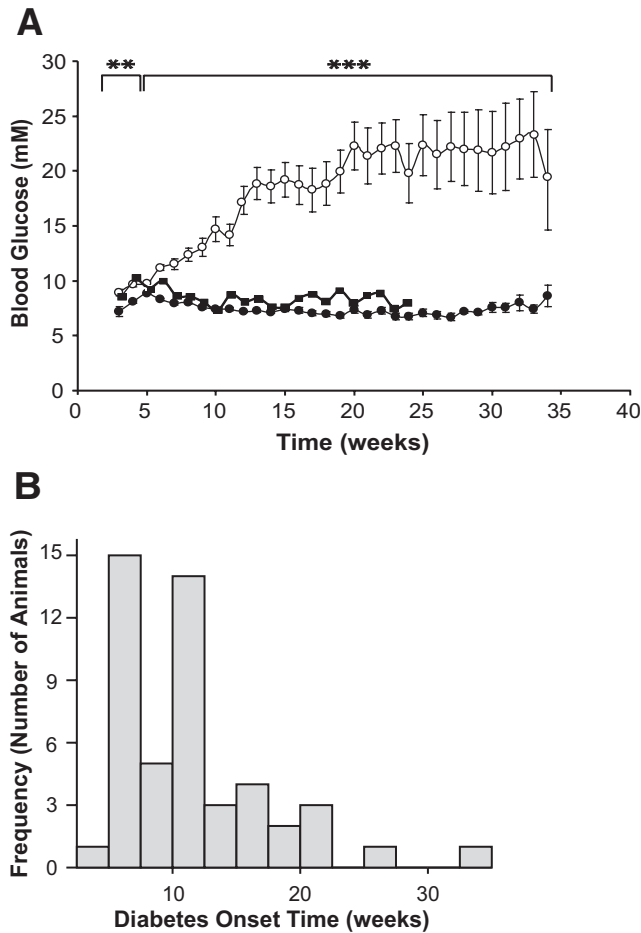


FIG. 1. Time-dependent changes in blood glucose and frequency of diabetes onset in human amylin transgenic mice. **A:** Blood glucose concentrations in human amylin transgenic mice (○, $n = 51$), matched nontransgenic controls (●, $n = 49$), and [^{25,28,29}Pro]human amylin transgenic mice (■, $n = 4$). **B:** Frequency of diabetes occurrence in the human amylin transgenic mice. Diabetes incidence peaked between 6 and 12 weeks of age, and cumulative incidence was 96%. ** $P < 0.01$, *** $P < 0.001$ for human amylin transgenic vs. nontransgenic mice.

transgenic mice before diabetes onset and during its progression, despite the high secretion rates of human amylin, shows that islet amyloid is not required for islet β -cell degeneration and loss of physiological insulin secretion. These findings are consistent with the reports of Janson et al. (19) and provide strong support for continuing exploration of the mechanism by which human amylin evokes β -cell death and contributes to the failure of insulin secretion in type 2 diabetes.

RESEARCH DESIGN AND METHODS

Generation of transgenic lines. Lines of human amylin transgenic mice were developed in a FVB/n genetic background through pronuclear microinjection of the following transgenic construct into fertilized oocytes.

The transgene construct consisted of a RT-PCR-generated human amylin cDNA under the regulatory control of the rat insulin-II promoter. Two lines (L13 and L9) were found to develop spontaneous diabetes. One of these, L13, was selected for intensive characterization and is that which is mainly described in this paper. Southern blotting of genomic DNA demonstrated that L13 hemizygous mice have $\sim 36 \pm 7$ copies of the transgene. Pancreas-specific expression of human amylin was confirmed by Northern blotting analysis of RNA transcripts from various organs.

[^{25,28,29}Pro]human amylin transgenic mice (L44) were generated in the FVB/n strain, as was L13, using a similar transgene construct with the wild-type human amylin cDNA replaced by a [^{25,28,29}Pro]human amylin cDNA. The modified human amylin variant has proline residues at positions 25, 28, and 29. This nonamyloidogenic human amylin transgenic line was generated

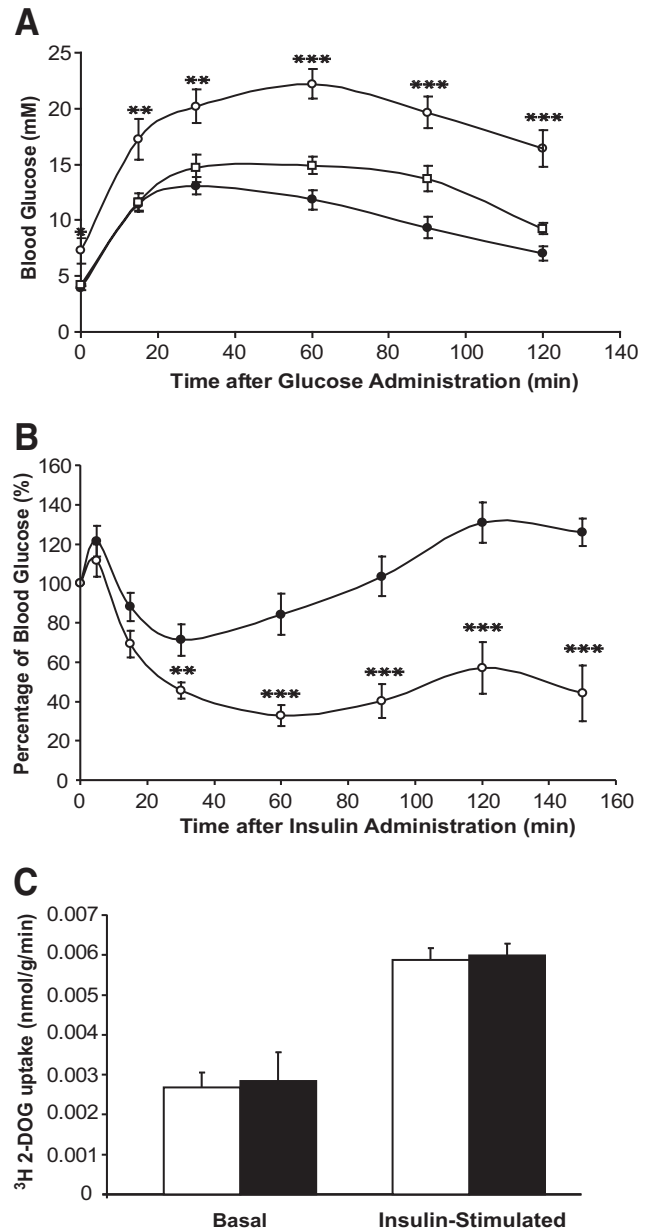


FIG. 2. Glucose tolerance, insulin tolerance, and muscle glucose uptake during diabetes progression in human amylin transgenic mice. **A:** IPGTT in human amylin transgenic (○, $n = 18$), [^{25,28,29}Pro]human amylin transgenic (□, $n = 4$), and nontransgenic control (●, $n = 16$) mice. **B:** IPITT in human amylin transgenic (○, $n = 15$) and nontransgenic littermates (●, $n = 14$). **C:** In vitro glucose uptake in soleus muscles incubated without (basal) and with 2 μ M insulin in fasted human amylin transgenic (■, $n = 7$) and nontransgenic controls (□, $n = 7$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

to provide a control to monitor the nonspecific effects of overexpression of a peptide within islet β -cells. Pancreas-specific expression of [^{25,28,29}Pro]human amylin was confirmed by Northern blotting and immunohistochemical analysis (results not shown).

Experimental design. Hemizygous L13 and L44 male animals were backcrossed with wild-type FVB/n females. Hemizygous male offspring were selected for this study, and nontransgenic male littermates were used as controls. Animals from L13 were divided into three groups based on weekly nonfasted blood glucose reading: pre-diabetic, mid-diabetic, and end-diabetic phases. Control animals and those from L44 were analyzed at similar time points. L13 animals that were normoglycemic at 3 and 4 weeks of age and those that had been hyperglycemic for 5 consecutive weeks were categorized into the pre- and mid-diabetic groups, respectively. End-diabetic animals were at the terminal stages of diabetes and displayed loss of exploratory behavior, relative immobility, and failure to groom. All animal experiments were approved by the institution's animal ethics committee.

TABLE 1

Pancreatic hormone levels at pre-, mid-, and end-diabetic stages in L13 human amylin transgenic, nontransgenic, and L44 [25,28,29Pro]human amylin transgenic (tritransgenic) mice

	Stage of diabetes		
	Pre-diabetic	Mid-diabetic	End-diabetic
Insulin			
Nontransgenic	160.6 ± 57.7	826.3 ± 162.7	718.8 ± 120.7
Transgenic	371.1 ± 99.7	195.8 ± 93.6*	21.3 ± 5.7†
Tritransgenic	746.7 ± 319.0*	2,509.4 ± 775.3*‡	2,675.3 ± 103.7†‡
Total amylin			
Nontransgenic	50.7 ± 5.2	78.2 ± 3.9	80.2 ± 6.6
Transgenic	128.8 ± 23.2*	27.8 ± 9.7†	5.2 ± 0.6†
Tritransgenic	318.1 ± 77.6†§	836.0 ± 17.0*‡	1,520.9 ± 505.9†‡
Human amylin			
Nontransgenic	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
Transgenic	14.3 ± 3.5†	7.3 ± 2.7¶	0.3 ± 0.1†
Mouse amylin			
Nontransgenic	50.7 ± 5.2	78.2 ± 3.9	80.2 ± 6.6
Transgenic	114.6 ± 19.9*	20.5 ± 7.0†	4.9 ± 0.6†
Glucagon			
Nontransgenic	33.8 ± 3.4	27.4 ± 3.7	22.6 ± 1.7
Transgenic	38.5 ± 6.0	27.9 ± 3.4	32.4 ± 4.0¶
Somatostatin			
Nontransgenic	5.2 ± 0.9	8.0 ± 0.7	6.4 ± 0.4
Transgenic	4.7 ± 0.9	6.9 ± 1.0	6.5 ± 0.9

Data are means ± SE. Hormone measurements (pmol/mg pancreatic protein) were normalized to total pancreatic protein before statistical analyses. * $P < 0.01$, † $P < 0.001$, and ¶ $P < 0.05$ vs. nontransgenic; ‡ $P < 0.001$ and § $P < 0.01$ vs. transgenic; $n = 10$.

Glucose and insulin tolerance tests. After an overnight fast, glucose was administered into the intraperitoneal cavity (1g/kg body wt). Blood glucose was determined using a glucose meter (Roche Diagnostics) before injection and at 5, 15, 30, 60, 90, and 120 min after injection. The procedure for the insulin tolerance test (ITT) was identical to that of the glucose tolerance test (GTT) with the exception that soluble insulin (Novo Nordisk Pharmaceuticals) was injected into the intraperitoneal cavity (1 mU/g body wt) after a 4-h fast.

Muscle glucose uptake assay. Soleus muscles were first preincubated for 30 min in Krebs-Henseleit bicarbonate buffer (KHB) supplemented with 8 mmol/l glucose and 32 mmol/l D-mannitol with or without 2 mU/ml insulin. Muscles were then rinsed and incubated in KHB buffer with 2 μ Ci/ml [³H]2-deoxyglucose (ICN Pharmaceuticals), 0.3 μ Ci/ml [¹⁴C]D-mannitol (ICN Pharmaceuticals), and 2 mmol/l sodium pyruvate for 30 min. All incubations were performed under 95% O₂/5% CO₂ with gentle agitation at 30°C followed by processing. Muscles were boiled (1 mol/l NaOH, 10 min), and muscle extracts were neutralized with 5 mol/l HCl. Radioactivity of muscle extracts and incubation media was determined in duplicates for dual labels.

Pancreatic protein analysis. Total protein was extracted from frozen pancreatic tissue by acid-ethanol (1.5% HCl, 75% ethanol, and 20 μ l/mg pancreatic wt). After overnight incubation (4°C on shaking), homogenates were centrifuged, supernatant was removed, and pancreatic extracts were neutralized (pH 7) with 1 mol/l Tris base. Total pancreatic protein was quantified using BCA assay (Sigma-Aldrich).

Hormone measurements. Immunoreactive insulin (Mercodia) and human amylin (Linco Research) were measured in plasma and pancreatic extracts using enzyme-linked immunosorbent assays (ELISAs). Total amylin (Peninsula Laboratories) and glucagon (Linco Research) were determined using radioimmunoassay (RIA) kits. Somatostatin was measured using an enzyme immunosorbent assay kit (Phoenix Pharmaceutical). All kits were used according to manufacturers' instructions.

Immunohistochemical and morphometric analyses. Pancreatic tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 7 μ m. Serial sections were stained with Congo red. Amyloid-positive pancreatic sections were included with every Congo red staining run as positive controls. Sections were also immunostained with anti-glucagon (ICN Pharmaceuticals), anti-insulin (Dako), anti-amylin (in-house derived), and/or anti-somatostatin (Santa Cruz Biotechnology) antibodies. Appropriate second antibodies conjugated to fluorescein isothiocyanate, Rhodamine Red, or Cy5 (Jackson ImmunoResearch Laboratories) were used for the covisualization of α -, β -, and δ -cells within the islets (TCS SP2; Leica Microsystems). Cross-sectional islet areas and total pancreatic section areas were determined in hematoxylin-eosin sections under light microscopy using image analyses software (Axio-

Vision 2; Carl Zeiss). β -Cell mass was estimated using a previously described method (24).

For immunofluorescence staining for cleaved caspase-3, pancreatic tissues were immediately frozen and embedded in OCT. Eight-micrometer sections were cut and initially blocked with 10% (vol/vol) normal goat serum for 2 h and then incubated with rabbit anti-cleaved caspase-3 (Cell Signaling) followed by detection using goat anti-rabbit Rhodamine Red-X-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). Stained sections were viewed and photographed using inverted-phase fluorescence microscopy (Nikon).

Transmission electron microscopy. Pancreases were cut into 1-mm³ pieces and fixed in 2% glutaraldehyde and 0.1 mol/l Sorenson's phosphate buffer (pH 7.2, 4°C, overnight). Specimens were postfixed with 0.1% osmium tetroxide and 0.1 mol/l Sorenson's phosphate buffer (pH 7.2, 1 h), followed by dehydration in increasing concentrations of ethanol solutions. Specimens were then infiltrated with a 1:1 mixture of epoxy resin and 100% ethanol (1 h, room temperature) followed by 100% epoxy resin infiltration overnight. Specimens were then embedded with fresh resin and cured (48 h, 60°C). Ultrathin sections were collected onto 200-mesh copper grids and double stained with 2% (wt/vol) aqueous uranyl acetate followed by lead citrate before viewing (CM-12; Phillips).

Statistical analyses. All data are expressed as means ± SE. Independent sample *t* tests were performed using SPSS v11.5. Two-way ANOVA, general linear model, and post hoc Tukey's honestly significant difference analyses were performed using Statistica 6.0. *P* values <0.05 were accepted as statistically significant.

RESULTS

Overexpression of human amylin leads to diabetes and glucose intolerance. Hemizygous human amylin transgenic mice developed hyperglycemia from 6 weeks after birth (Fig. 1A), from which time blood glucose values were elevated compared with controls ($P < 0.01$). Blood glucose remained significantly elevated throughout the entire 31-week monitoring period (Fig. 1A). Ninety-six percent of the transgenic mice developed diabetes during this monitoring period, with a mean onset time of 11.2 weeks (Fig. 1B). Intraperitoneal GTTs (IPGTTs) performed in fasted animals at the mid-diabetic stage (Fig. 2A) demonstrated impaired glucose tolerance and ele-

vated fasting blood glucose (Fig. 2A) in human amylin transgenic mice ($P < 0.05$). The lack of glucose-stimulated insulin secretion during GTT present in human amylin transgenic mice (t_0 29.9 ± 15.1 cf. t_{15} 39.1 ± 18.5 pmol/l; NS), is consistent with impaired β -cell function. In contrast, we detected a positive insulin response to glucose in the control nontransgenic mice (t_0 26.2 ± 4.3 cf. t_{15} 128.6 ± 55.7 pmol/l; $P < 0.05$). Neither fasting hyperglycemia nor impaired glucose tolerance was present in [^{25,28,29}Pro]human amylin transgenic mice (Fig. 2A), wherein blood glucose concentrations remained equivalent to control levels at all times during the observation period (from birth to 24 weeks of age) (Fig. 1A).

Systemic insulin administration (intraperitoneal ITT [IP-ITT]) at the mid-diabetic stage (Fig. 2B) showed that human amylin transgenic mice were more responsive to insulin, consistent with the absence of peripheral insulin resistance ($P < 0.01$). Absence of peripheral insulin resistance in human amylin transgenic mice was confirmed by ex vivo glucose uptake analysis in isolated, stripped soleus muscles (Fig. 2C), which showed similar rates of glucose uptake between transgenic and nontransgenic animals in either the basal or the insulin-stimulated states. This finding is consistent with that from the ITT analysis and confirms that peripheral insulin resistance is not implicated in the pathogenesis of diabetes in this model.

Human amylin overexpression leads to decreased islet function. Plasma and pancreatic hormone levels were determined at the pre-diabetic, mid-diabetic, and end-diabetic phases and at equivalent times in nondiabetic mice. Elevated pancreatic human amylin ($P < 0.001$) and, hence, total amylin ($P < 0.01$) was present before diabetes developed (Table 1). The development of diabetes in these mice (at the mid- and end-diabetic stages) was associated with concomitant decrements in pancreatic β -cell hormones: insulin ($P < 0.01$) and total amylin ($P < 0.001$). By contrast, in [^{25,28,29}Pro]human amylin transgenic mice, which did not develop diabetes, the pancreatic total amylin and insulin levels were significantly higher than in nondiabetic animals at all three phases (Table 1). This suggests that overexpression of human amylin results in β -cell destruction and impairment of β -cell function, which is consistent with our histological evidence of β -cell loss during diabetes progression (Fig. 3A–F). The decrease in β -cell mass in human amylin transgenic mice (Fig. 3H) confirms that the loss of β -cell mass contributes to the mechanism of diabetes in these human amylin mice, at least in part. Pancreatic glucagon was also elevated at the end-diabetic stage in transgenic animals ($P < 0.05$), whereas no significant alteration in pancreatic somatostatin was observed (Table 1).

Plasma hormone concentrations also showed responses similar to those of their respective pancreatic contents (Table 2). As expected, human amylin was detected in plasma of transgenic animals only (at the pre-, mid-, and end-diabetic phases; $P < 0.05$ in each case). At the end-diabetic stage, plasma insulin was decreased in transgenic mice ($P < 0.05$); by contrast, increased plasma glucagon was observed in transgenic animals at this stage ($P < 0.001$). Plasma total amylin and somatostatin could not be determined because of insufficient sample volumes.

The specificity of the amylin assay systems used in this study enabled us to calculate estimated values for pancreatic mouse amylin by subtraction of values for pancreatic human amylin from corresponding measurements of total pancreatic amylin (Table 1). Both the total amylin RIA and

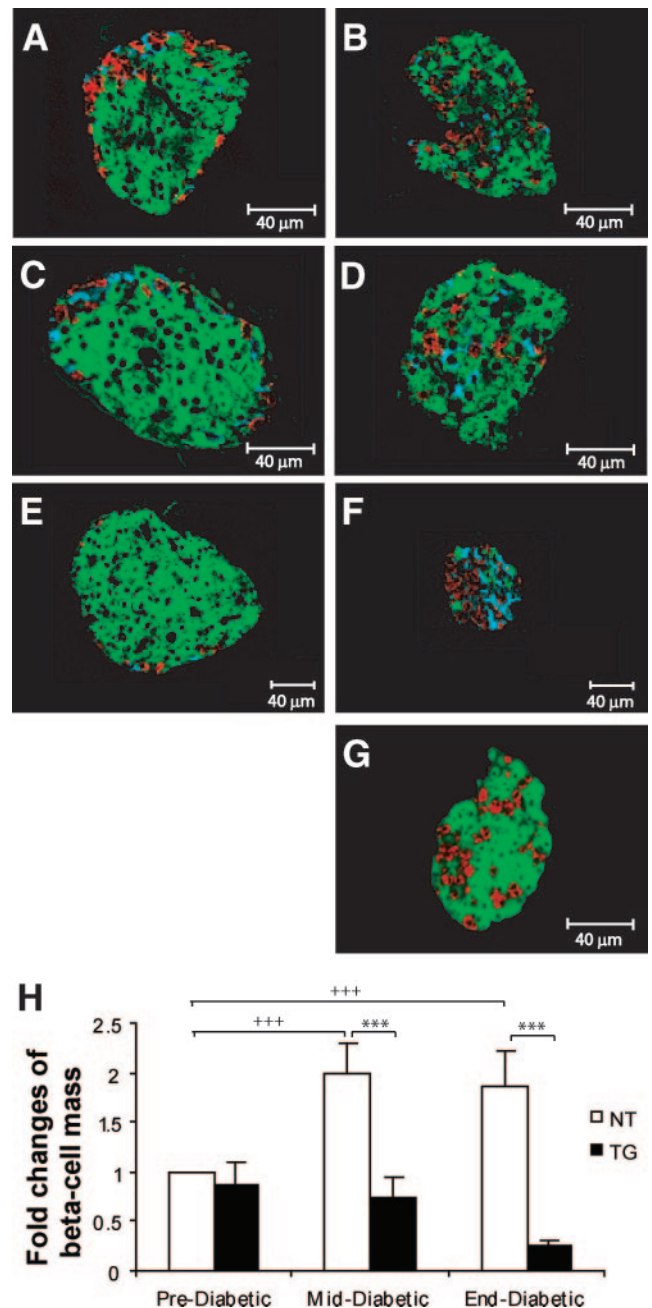


FIG. 3. Expression patterns of insulin, glucagon, and somatostatin and quantitative histomorphometric analysis in pancreatic islets from transgenic and nontransgenic mice. Representative sections of insulin (green), glucagon (red), and somatostatin (cyan) immunolocalization in nontransgenic control (A, C, and E) and human amylin transgenic (B, D, and F) mice at pre-diabetic (A and B), mid-diabetic (C and D), and late-diabetic (E and F) stages. G: Islet hormone expression from a late-stage [^{25,28,29}Pro]human amylin transgenic mouse. H: β -Cell mass analysis from human amylin transgenic and nontransgenic animals. β -Cell mass is estimated from data for the total pancreatic weight and the relative volume (derived from percentage of the islet area) of the β -cells, as described by Bonner-Weir (24). The pancreatic weight is equated to pancreatic volume with the reasonable assumption that 1 cm³ tissue weighs 1 g. Results are expressed as fold changes of β -cell mass as relative to nontransgenic mice at the pre-diabetic stage, whose numbers were set at unity. +++ $P < 0.001$, nontransgenic mid-diabetic/end-diabetic vs. nontransgenic pre-diabetic. *** $P < 0.001$, transgenic vs. nontransgenic. (Please see <http://dx.doi.org/10.2337/db06-1755> for a high-quality digital representation of this image.)

the human amylin ELISA systems were validated to confirm that antibodies in each recognized the appropriate amylin species. Our validation experiments demonstrated

TABLE 2
Plasma hormone levels at the pre-, mid-, and end-diabetic stages in human amylin transgenic and nontransgenic animals

	Stage of diabetes		
	Pre-diabetic	Mid-diabetic	End-diabetic
Insulin			
Nontransgenic	120.5 ± 40.4	176.2 ± 36.8	178.4 ± 46.9
Transgenic	78.3 ± 21.9	104.2 ± 31.5	10.0 ± 5.3*
Human amylin			
Nontransgenic	0.7 ± 0.4	1.2 ± 0.2	2.1 ± 0.3
Transgenic	27.7 ± 7.9†	14.9 ± 3.8*	3.7 ± 0.01*
Glucagon			
Nontransgenic	22.6 ± 3.1	14.0 ± 4.3	11.9 ± 1.5
Transgenic	33.0 ± 7.2	15.6 ± 3.5	43.0 ± 5.1‡

Data are means ± SE (pmol/l). * $P < 0.05$, † $P < 0.01$, and ‡ $P < 0.001$; nontransgenic vs. transgenic; $n = 10$.

that antibodies used in the ELISA specifically detected human amylin and did not cross-react with mouse amylin (Fig. 4A). Antibodies used in the RIA system were 100% cross-reactive with human amylin, murine amylin, and [^{25,28,29}Pro]human amylin, thus recognizing human and murine amylin at equimolar concentrations but detecting [^{25,28,29}Pro]human amylin at lower sensitivity compared with the former (Fig. 4B). Antibodies in both assay sys-

tems also required that amylin be amidated at its COOH terminus for recognition.

Elevated levels of mouse amylin were observed in transgenic animals before diabetes onset ($P < 0.01$). Thus, overexpression of human amylin may have contributed to the elevated production of endogenous amylin. However, decreased pancreatic mouse amylin concentrations were present at the mid- and end-diabetic stages in human amylin transgenic mice, consistent with the progressive decline in β -cell function that occurred during the course of diabetes progression in these animals.

Abnormal islet architecture in the absence of islet amyloid formation. Immuno-labelling of α -, β -, and δ -cells at the end-diabetic stage showed markedly decreased β -cell numbers in transgenic islets, consistent with decreased numbers of functional β -cells and decreased insulin production in these animals (Fig. 3F). Electron microscopic studies, in which at least 30 islets were examined from each animal, further confirmed the presence of typical β -cell secretory granules in nondiabetic islets (Fig. 5G) that, by contrast, were largely lacking in those β -cells (defined as endocrine cells present in the body of the pancreas but lacking typical α - or δ -cell granules) remaining in the body of the pancreas in human amylin transgenic mice at the end stage (Fig. 5H).

β -Cells from end-stage diabetic mice frequently showed invagination of their nuclear membranes and chromatin margination, both characteristics of cells undergoing apoptosis (Fig. 5H). In addition, apoptosis was also detected and quantified by immunofluorescence staining for cleaved caspase-3 (Fig. 5I and J). About 60 islets from each study group (20 islets per animal and 3 animals per group) were examined, and the total number of cleaved caspase-3-positive cells was determined. Numbers of apoptotic islet cells were found to be significantly increased in the transgenic mice at the mid-diabetic stage (Fig. 5I) compared with matched nondiabetic, nontransgenic mice (Fig. 5K). These findings are consistent with *in vitro* studies demonstrating human amylin-evoked apoptosis in cultured β -cells (15). In contrast, equivalent β -cell loss was never observed in islets of [^{25,28,29}Pro]human amylin transgenic mice, although alterations in the relative positioning of α - and β -cells similar to those demonstrated in human amylin transgenic mice were present (Fig. 3G). This latter observation indicates that the modifications in islet cell distribution were insufficient in themselves to cause β -cell loss or defective insulin secretion.

Examination under polarized light of Congo red-stained sections revealed the absence of green birefringence at all three stages of the diabetic process in human amylin transgenic mice (Fig. 5A–F). Moreover, Congo red-stained islet amyloid was never detected in [^{25,28,29}Pro]human amylin transgenic mice (data not shown). These findings indicate that Congo red-stainable islet amyloid is not necessary for and cannot have played a role in the development of diabetes in this model. We note that islet amyloid-positive sections as positive controls were included with every Congo red staining run.

DISCUSSION

Here, we report characterization of a hemizygous line of human amylin transgenic mice (L13) that synthesizes human amylin in its pancreatic islets and demonstrates increased release of human amylin into the plasma (Table 2). In the current study, 96% of L13 transgenic mice

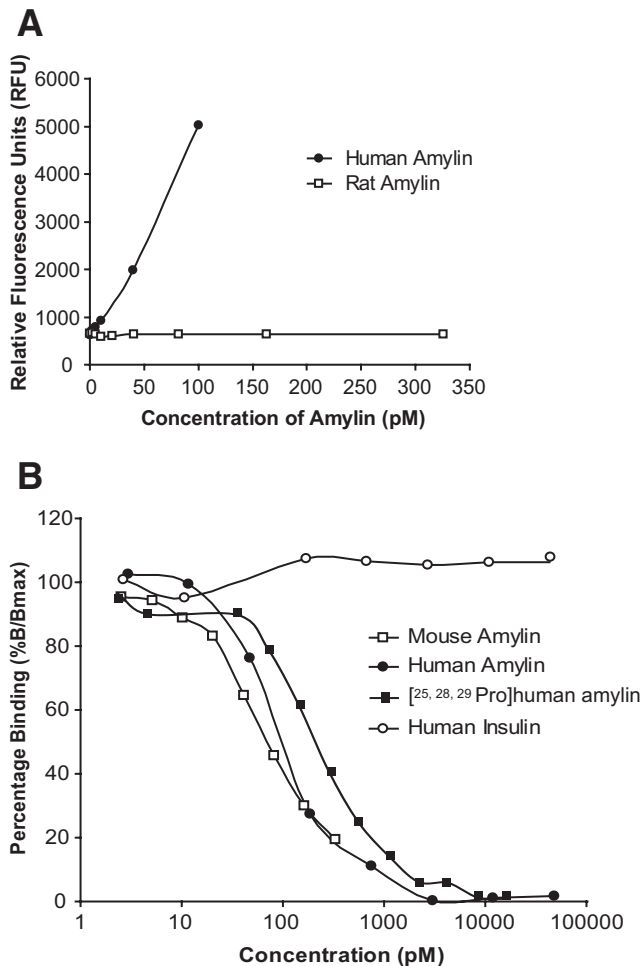


FIG. 4. Antibody cross-reactivity against human amylin and murine amylin measured in ELISA (A) and RIA (B) systems. ●, Human amylin; □, murine amylin; ■, [^{25,28,29}Pro]human amylin; and ○, human insulin.

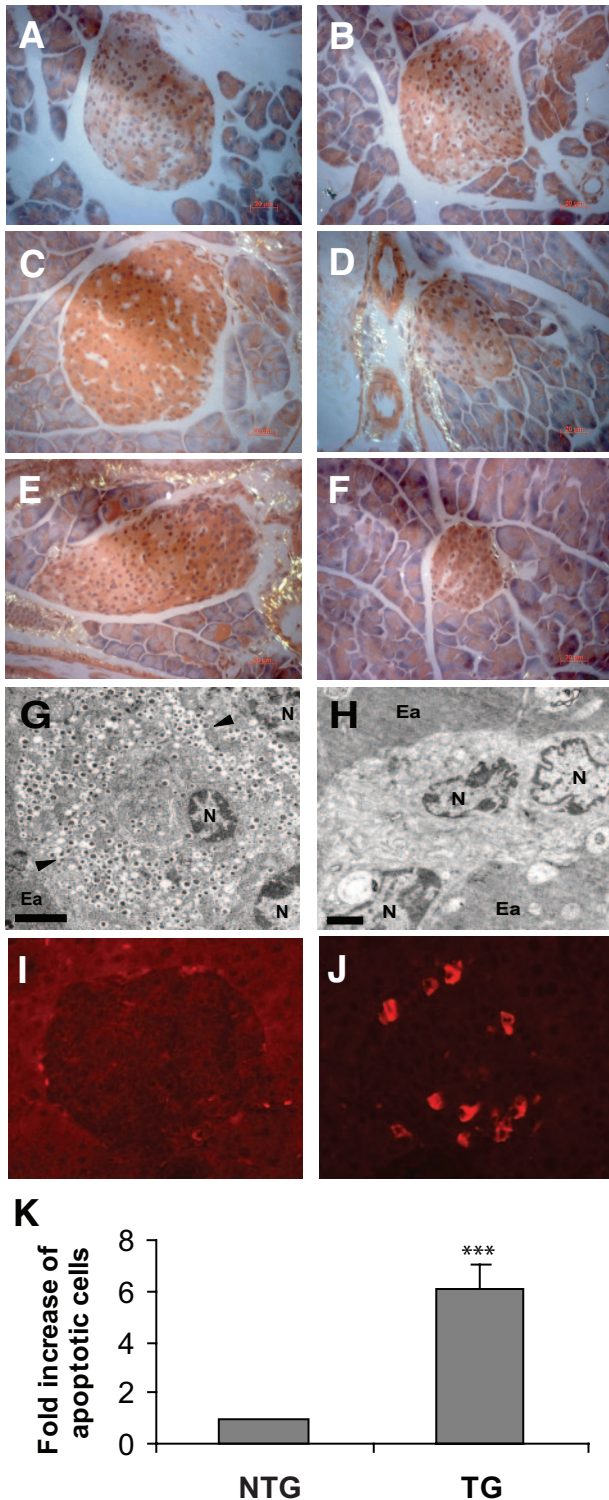


FIG. 5. Light and electron microscopic analysis of structure and immunofluorescence study of apoptosis in islets from representative nontransgenic (NTG) and human amylin transgenic (TG) mice. Congo red-stained section imaged under polarized light of representative islets from nontransgenic (*A*, *C*, and *E*) and transgenic (*B*, *D*, and *F*) mice at pre-diabetic (*A* and *B*), mid-diabetic (*C* and *D*), and late-diabetic (*E*, *F*) stages. Birefringence is collagenous. Magnification $\times 400$. TEM images of islets from end-stage nontransgenic (*G*) and human amylin transgenic (*H*) mice. *G*: Normal β -cell insulin secretory granules with hallmark dense cores and surrounding halos (arrowed), healthy nucleus (N), and surrounding exocrine acini (Ea). *H*: Section from end-stage diabetic human amylin transgenic mouse showing characteristic absence of insulin secretory granules and margination of chromatin with invagination of the nuclear membrane typical of apoptosis and surrounding exocrine acini. Ea, endocrine acini; N, nucleus. Scale bars = 2 μ m.

developed diabetes (sustained hyperglycemia, Fig. 1A) and $\sim 95\%$ were diabetic by 22.5 weeks of age; by contrast, corresponding rates of diabetes in nontransgenic littermates were 0% (Fig. 1). Diabetes in L13 animals is associated with decreased β -cell function (Table 1). These animals have never been found to be hyperinsulinemic (Table 2) but show significant impairment of glucose tolerance (Fig. 2A) in the absence of peripheral insulin resistance (Fig. 2C) or islet amyloid formation (Fig. 5A–F).

L13 transgenic mice had unaltered levels of pancreatic and plasma insulin during the pre-diabetic stage (Tables 1 and 2). Despite the lack of significant differences in plasma insulin between transgenic and nontransgenic mice at the mid-diabetic stage (Table 2), a fourfold decrease in pancreatic insulin was present in transgenic mice during this period (Table 1). At the end-diabetic stage, however, >17 -fold decreases in pancreatic and plasma insulin were present (Tables 1 and 2). The progressive development of β -cell dysfunction over the diabetic process was paralleled by a progressive loss of insulin immunoreactive cells between the mid- (Fig. 3D) and end-diabetic stages (Fig. 3F) in transgenic islets, indicating progressive falls in the number of functional β -cells in hemizygous L13 mice. The disappearance of islet β -cells with concomitant development of impaired insulin secretion is thus responsible for the observed diabetic phenotype in this model.

Lines of [25,28,29 Pro]human amylin transgenic mice were also constructed to address the possible nonspecific causation of diabetes by overexpression of protein within islet β -cells. The aggregation potential of [25,28,29 Pro]human amylin has been eliminated by substitution of three prolyl residues at positions 25, 28, and 29 within the wild-type human amylin molecule. Mice expressing the [25,28,29 Pro]human amylin transgene in their islet β -cells do not develop either islet β -cell disappearance or insulin-deficient diabetes (Fig. 3G), demonstrating that the insertion of this transgene and its expression in islet β -cells of a nonfibrillogenic amylin variant is not in itself sufficient to cause β -cell destruction. Given that [25,28,29 Pro]human amylin mice expressing a nonaggregating amylin variant do not develop diabetes (Fig. 2A) or islet β -cell degeneration (Fig. 3G), findings presented here support the idea that the development of diabetes in mice expressing wild-type human amylin (L13) is caused specifically by the expression of a fibrillogenic amylin variant in islet β -cells rather than simply a nonspecific event caused by overexpression of any protein from this transgene in this location. It thus appears that the amyloidogenic property of human amylin is necessary for its ability to cause β -cell disappearance and diabetes, just as it is for its *in vitro* cytotoxicity (25,26).

Human amylin transgenic mouse models have previously been investigated by other laboratories (16–19). Phenotypic observations reported in those mice have been wide ranging; these have varied from a complete lack of spontaneous diabetes (16–18) to the appearance of severe early-onset diabetes in one line (19). The data reported here are closest to those reported by Janson et al. (19) and

Immunofluorescence staining for cleaved caspase-3 in nondiabetic nontransgenic mice (*I*) and mid-diabetic transgenic mice (*J*). *K*: Quantitative measurement of islet cell apoptosis by numeric counts of cleaved caspase-3-positive cells. Results are expressed as fold increase of apoptotic cells in transgenic mice compared with nontransgenic mice, whose numbers were set at unity. *** $P < 0.001$ vs. nontransgenic. (Please see <http://dx.doi.org/10.2337/db06-1755> for a high-quality digital representation of this figure.)

show the presence of early-onset diabetes without the formation of detectable islet amyloid. However, our findings were demonstrated in hemizygous animals, whereas those of Janson et al. (19) were reported in homozygous mice, although it has yet to be determined whether these two lines have comparable human amylin expression levels. Differences in amylin expression levels are one possible explanation for phenotypic variation between these strains of transgenic mice.

Reported phenotypic differences between strains of human amylin transgenic mice might also be attributable, at least in part, to the genetics of the background strain in which they were generated. Such transgenic mice have mainly been developed in two genetic backgrounds, FVB/n, in the current study and that of Janson et al. (19), and C57BL/6J (16,18). Severe spontaneous diabetes has previously been reported in homozygous individuals from a line generated in the FVB/n background (19) but has reportedly been absent in C57BL/6J-based strains (16,18). The influence of genetic background on phenotypic outcomes is well recognized. For example, Haluzik et al. (2004) reported the effect of the *ob/ob* mutation in both FVB/n and C57BL/6J backgrounds. Mice from the FVB/n strain were severely hyperglycemic, had lower triglyceride clearance, and were more insulin resistant than their C57BL/6J counterparts (27). This implies that the FVB/n background could be more susceptible to abnormal lipid and glucose metabolism and thus might have a greater predisposition to the development of diabetes. The L13 transgenic mouse model reported here was generated in the FVB/n background and demonstrated development of spontaneous diabetes, consistent with this hypothesis.

Previous *in vitro* studies are also consistent with the idea that the cytotoxicity of amyloid-forming amylin peptides, such as human amylin *per se*, is conferred by soluble species rather than mature, aggregated fibrils (2,25,28). Activation of an apoptotic JNK1/c-Jun/caspase-8/caspase-3 pathway is a related downstream mechanism by which amylin-induced destruction of islet β -cells occurs *in vitro* (29–31). Here, we have shown that caspase-3 activation also occurs in pancreatic islets of L13 hemizygous mice but not in the islets of their nontransgenic littermates; both findings are consistent with β -cell destruction through such an apoptotic pathway in these transgenic mice. Although both non- β -cells and β -cells could theoretically have contributed to our analysis of islet cell apoptosis by activated caspase 3 immunofluorescence, we believe that it was mostly due to β -cells because of the known β -cell targeting properties of the human amylin construct used herein and the demonstrated loss of β -cells from the islets of diabetic transgenic animals, whereas non- β -cells (glucagon- and somatostatin-containing) were relatively increased in the islets of late-diabetic animals (Fig. 3F). In addition, transmission electron microscopy (TEM) findings reported herein also provide structural evidence for the occurrence of β -cell apoptosis in these transgenic mice (Fig. 5H), consistent with other reports (2,15,32).

Despite the overexpression of human amylin in pancreatic islet β -cells, Congo red-stainable islet amyloid did not contribute to the pathogenesis of diabetes in L13 transgenic mice (Fig. 5A–F). However, we cannot exclude the possibility of a presence of small human amylin aggregates/oligomers that is not detectable by Congo red staining. An absence of islet amyloid has previously been reported in another line of human amylin transgenic mice that develop spontaneous diabetes (19). The greater than

twofold increase in pancreatic mouse amylin observed in L13 mice (Table 1) may play a role in the lack of islet amyloid formation in these mice. Westermarck et al. (2000) reported that mice expressing human amylin without murine amylin ($hA^{+/+}/mA^{-/-}$) developed extracellular fibrillar amyloid deposits by 12 months of age, compared with transgenic human amylin mice ($hA^{+/+}/mA^{+/+}$), which had developed them by 16 months of age. The earlier onset of islet amyloid formation in $hA^{+/+}/mA^{-/-}$ mice compared with $hA^{+/+}/mA^{+/+}$ mice was reportedly interpreted as consistent with an inhibitory effect of mouse amylin on fibril formation (33).

In summary, islet-specific overexpression of human amylin in L13 mice caused diabetes associated with degeneration and loss of islet β -cells with progressive impairment of insulin secretion. The disease mechanism in these mice is independent of both large islet amyloid deposits and peripheral insulin resistance. Overexpression of both human amylin and [^{25,28,29}Pro]human amylin caused altered islet-cell type distribution, consistent with prior reports (34). The pathophysiological relevance of this alteration is unclear, but it may be a nonspecific effect of overexpression of a protein in β -cells and is unlikely in itself to be causative of impaired insulin secretion. When combined, these two lines of transgenic mice form a composite model that may well have utility in the search for compounds with the pharmacological properties of ameliorating or suppressing human amylin-mediated β -cell death and type 2 diabetes evoked by human amylin-mediated mechanisms.

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REFERENCES

1. Maclean N, Ogilvie RF: Quantitative estimation of the pancreatic islet tissue in diabetic subjects. *Diabetes* 4:367–376, 1955
2. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC: β -Cell deficit and increased β -cell apoptosis in humans with type 2 diabetes. *Diabetes* 52:102–110, 2003
3. Deng S, Vatamaniuk M, Huang X, Doliba N, Lian MM, Frank A, Velidedeoglu E, Desai NM, Koeberlein B, Wolf B, Barker CF, Naji A, Matschinsky FM, Markmann JF: Structural and functional abnormalities in the islets isolated from type 2 diabetic subjects. *Diabetes* 53:624–632, 2004
4. Cooper GJS, Willis AC, Clark A, Turner RC, Sim RB, Reid KBM: Purification and characterisation of a peptide from amyloid-rich pancreases of type 2 diabetic patients. *Proc Natl Acad Sci U S A* 84:8628–8632, 1987
5. Westermarck P, Wernstedt C, Wilander E, Hayden DW, O'Brien TD, Johnson KH: Amyloid fibrils in human insulinoma and islets of Langerhans of the diabetic cat are derived from a neuropeptide-like protein also present in normal islet cells. *Proc Natl Acad Sci U S A* 84:3881–3885, 1987
6. Glenner GG, Eanes DE, Wiley CA: Amyloid fibrils formed from a segment of the pancreatic islet amyloid protein. *Biochem Biophys Res Commun* 155:608–614, 1988
7. Bell ET: Hyalinization of the islet of Langerhans in diabetes mellitus. *Diabetes* 1:341–344, 1952

8. Bell ET: Hyalinization of the islets of Langerhans in non-diabetic individuals. *Am J Pathol* 35:801–805, 1959
9. Clark A, Cooper GJS, Lewis CE, Willis AC, Morris JF, Reid KBM, Turner RC: Islet amyloid formed from diabetes-associated peptide may be pathogenic in type 2 diabetes. *Lancet* 330:231–234, 1987
10. Westermark P, Grimelius L: The pancreatic islet cells in insular amyloidosis in human diabetic and non-diabetic adults. *Acta Pathol Micro Scand A* 81:291–300, 1973
11. Zhao HL, Lai FM, Tong PC, Zhong DR, Yang D, Tomlinson B, Chan JC: Prevalence and clinicopathological characteristics of islet amyloid in Chinese patients with type 2 diabetes. *Diabetes* 52:2759–2766, 2003
12. Howard CF Jr: Longitudinal studies on the development of diabetes in individual *Macaca nigra*. *Diabetologia* 29:301–306, 1986
13. Johnson KH, O'Brien TD, Jordan K, Westermark P: Impaired glucose tolerance is associated with increased islet amyloid polypeptide (IAPP) immunoreactivity in pancreatic beta cells. *Am J Pathol* 135:245–250, 1989
14. Lorenzo A, Razzaboni B, Weir GC, Yankner BA: Pancreatic islet cell toxicity of amylin associated with type-2 diabetes mellitus. *Nature* 368:756–760, 1994
15. Saafi EL, Konarkowska B, Zhang S, Kistler J, Cooper GJS: Ultrastructural evidence that apoptosis is the mechanism by which human amylin evokes death in RINm5F pancreatic islet β -cells. *Cell Biol Int* 25:339–350, 2001
16. D'Alessio DA, Verchere CB, Kahn SE, Hoagland V, Baskin DG, Palmiter RD, Ensink JW: Pancreatic expression and secretion of human islet amyloid polypeptide in transgenic mice. *Diabetes* 43:1457–1461, 1994
17. Fox N, Schrementi J, Nishi M, Ohagi S, Chan SJ, Heisserman JA, Westermark GT, Leckstrom A, Westermark P, Steiner DF: Human islet amyloid polypeptide transgenic mice as a model of non-insulin-dependent diabetes mellitus (NIDDM). *FEBS Lett* 323:40–44, 1993
18. Hoppener JWM, Verbeek JS, de Koning EJP, Oosterwijk C, van Hulst KL, Visser-Vernooy HJ, Hofhuis E, van Gaalen S, Berends MJH, Hackeng WHL, Jansz HS, Morris JF, Clark A, Capel PJA, Lips CJM: Chronic overproduction of islet amyloid polypeptide/amylin in transgenic mice: lysosomal localisation of human islet amyloid polypeptide and lack of marked hyperglycemia or hyperinsulinaemia. *Diabetologia* 36:1258–1265, 1993
19. Janson J, Soeller WC, Roche PC, Nelson RT, Torchia AJ, Kreutter DK, Butler PC: Spontaneous diabetes mellitus in transgenic mice expressing human islet amyloid polypeptide. *Proc Natl Acad Sci U S A* 93:7283–7288, 1996
20. Couce M, Kane LA, O'Brien TD, Charlesworth J, Soeller W, McNeish J, Kreutter D, Roche P, Butler PC: Treatment with growth hormone and dexamethasone in mice transgenic for human islet amyloid polypeptide causes islet amyloidosis and β -cell dysfunction. *Diabetes* 45:1094–1101, 1996
21. Soeller WC, Janson J, Hart SE, Parker JC, Carty MD, Stevenson RW, Kreutter DK, Butler PC: Islet amyloid-associated diabetes in obese *Ay/a* mice expressing human islet amyloid polypeptide. *Diabetes* 47:743–750, 1998
22. Verchere CB, D'Alessio DA, Palmiter RD, Weir GC, Bonner-Weir S, Baskin DG, Kahn SE: Islet amyloid formation associated with hyperglycemia in transgenic mice with pancreatic β -cell expression of human islet amyloid polypeptide. *Proc Natl Acad Sci U S A* 93:3492–3496, 1996
23. Butler AE, Jang J, Gurlo T, Carty MD, Soeller WC, Butler PC: Diabetes due to a progressive defect in β -cell mass in rats transgenic for human islet amyloid polypeptide (HIP rat). *Diabetes* 53:1509–1516, 2004
24. Bonner-Weir S: Beta-cell turnover: its assessment and implications. *Diabetes* 50 (Suppl. 1):S20–S24, 2001
25. Konarkowska B, Aitken JF, Kistler J, Zhang S, Cooper GJS: The aggregation potential of human amylin determines its cytotoxicity towards islet β -cells. *FEBS J* 273:3614–3624, 2006
26. Zhang S, Liu J, Dragunow M, Cooper GJS: Fibrillogenic amylin evokes islet β -cell apoptosis through linked activation of a caspase cascade and JNK-1. *J Biol Chem* 278:52810–52819, 2003
27. Haluzik M, Colombo C, Gavrilova O, Chua S, Wolf N, Chen M, Stannard B, Dietz KR, Le Roith D, Reitman ML: Genetic background (C57BL/6J versus FVB/N) strongly influences the severity of diabetes in insulin resistance in *ob/ob* mice. *Endocrinology* 145:3258–3264, 2004
28. Porat Y, Kolusheva S, Jelinek R, Gazit E: The human islet amyloid polypeptide forms transient membrane-active prefibrillar assemblies. *Biochem* 42:10971–10977, 2003
29. Rumora L, Hadzija M, Barisic K, Maysinger D, Grubisic TZ: Amylin-induced cytotoxicity is associated with activation of caspase-3 and MAP kinases. *Biol Chem* 383:1751–1758, 2002
30. Zhang S, Liu H, Liu J, Tse CA, Dragunow M, Cooper GJS: Activation of activating transcription factor 2 by p38 MAP kinase during apoptosis induced by human amylin in cultured pancreatic β -cells. *FEBS J* 273:3779–3791, 2006
31. Zhang S, Liu J, Saafi EL, Cooper GJS: Induction of apoptosis by human amylin in RINm5F islet β -cells is associated with enhanced expression of p53 and p21. *FEBS Lett* 455:315–320, 1999
32. Butler AE, Janson J, Soeller WC, Butler PC: 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. *Diabetes* 52:2304–2314, 2003
33. Westermark GT, Gebre-Medhin S, Steiner DF, Westermark P: Islet amyloid development in a mouse strain lacking endogenous islet amyloid polypeptide (IAPP) but expressing human IAPP. *Mol Med* 6:998–1007, 2000
34. Wong HY, Ahren B, Lips CJM, Hoppener JWM, Sundler F: Postnatally disturbed pancreatic islet cell distribution in human islet amyloid polypeptide transgenic mice. *Regul Peptides* 113:89–94, 2003