Spontaneous Diabetes in Hemizygous Human Amylin Transgenic Mice that developed neither Islet Amyloid nor Peripheral Insulin Resistance

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Objectives: (1) Determine whether soluble-misfolded amylin or insoluble-fibrillar amylin may cause, or instead result from diabetes, in human amylin transgenic mice, and (2) the role, if any, that insulin resistance might play in these processes.

Methods: We characterised the phenotypes of independent transgenic mouse lines that display pancreas-specific expression of human amylin or a non-aggregating homolog, [25,28,29Pro]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, since 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 non-transgenic mice, glucagon- and somatostatin-cells were present mainly at the periphery and insulin-cells were mainly in the core, whereas by contrast all three cell types were distributed throughout the islet in transgenic animals. [25,28,29Pro]human amylin transgenic mice developed neither β-cell degeneration nor glucose intolerance.

Conclusions: Over-expression 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.
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 (IAPP), 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 to 90% of pancreases from type-2 diabetic subjects studied at 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 characterisation 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 over-expression 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 (1996) 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 over-expression of human amylin is sufficient for diabetes development but not islet amyloid formation in that model. It was previously thought that over-expression 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, due at least in part 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., 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 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 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 pro-nuclear microinjection of the following transgenic construct into fertilised oocytes.

<table>
<thead>
<tr>
<th>Rat Insulin II Promot</th>
<th>human amylin cDNA</th>
<th>human albumin Intron I</th>
<th>human GAPDH 3'-UTR</th>
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<tr>
<td>877 bp</td>
<td>271 bp</td>
<td>723 bp</td>
<td>523 bp</td>
</tr>
<tr>
<td>Xhol</td>
<td>Ncol</td>
<td>Clal</td>
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The transgene construct consisted of a RT-PCR-generated human amylin cDNA under the regulatory control of the rat insulin-II promoter as illustrated above. Two lines (L13 and L9) were found to develop spontaneous diabetes. One of these, L13, was selected for intensive characterisation and is that which is mainly described in this paper. Southern blotting of genomic DNA demonstrated that L13 hemizygous mice have approximately 36 ± 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 also generated in the FVB/N strain as 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 non-amyloidogenic human amylin transgenic line was generated to provide a control to monitor the non-specific effects of over-expression 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 non-transgenic male littermates were used as controls. Animals from L13 were divided into three groups based on weekly non-fasted blood glucose reading; pre-diabetic, mid-diabetic and end-diabetic phases, and control animals and those from L44 were analysed 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 categorised into the pre- and mid-diabetic groups respectively. End-diabetic animals were at the terminal stages of diabetes and displayed loss of exploratory behaviour, relative immobility and failure to groom. All animal experiments were approved by the institution’s animal ethics committee.

Glucose and Insulin Tolerance Tests. Following an overnight fast, glucose was administered into the intraperitoneal cavity (1g/kg body weight). Blood glucose was determined using a glucose meter (Roche Diagnostics) before injection and at 5, 15, 30, 60, 90 and 120 minutes post-injection. The procedure for the insulin tolerance test was identical to that of glucose tolerance test with the exception that soluble insulin (Novo Nordisk Pharmaceuticals) was injected into the intraperitoneal cavity (1mU/g body weight) following a 4 hour fast.

Muscle Glucose Uptake Assay. Soleus muscles were first pre-incubated for 30 minutes in Krebs-Henseleit Bicarbonate buffer (KHB) supplemented with 8mM glucose and 32mM D-mannitol with or without 2mU/ml insulin. Muscles were then rinsed and incubated in KHB buffer with 2μCi/ml [^3H]-2-deoxyglucose (ICN Pharmaceuticals), 0.3μCi/ml [^14C]-D-
mannitol (ICN Pharmaceuticals), 2mM sodium pyruvate for 30 minutes. All incubations were performed under 95%O2-5%CO2 with gentle agitation at 30°C followed by processing. Muscles were boiled (1M NaOH, 10 minutes) and muscle extracts neutralised with 5M 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, 20μl/mg pancreatic weight). Following overnight incubation (4°C on shaking), homogenates were centrifuged and Supernatant was removed and pancreatic extracts were neutralised (pH 7) with 1M Tris Base. Total pancreatic protein was quantified using BCA assay (Sigma-Aldrich).

**Hormone Measurements.** Immunoreactive insulin (Mercodia AB) and human amylin (Linco Research, USA) were measured in plasma and pancreatic extracts using ELISA assays. Total amylin (Peninsula Laboratories) and glucagon (Linco Research) were determined using RIA kits. Somatostatin was measured using an EIA 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 7μm. 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 Corporation), anti-amylin (in-house derived) and/or anti-somatostatin (Santa Cruz Biotechnology) antibodies. Appropriate second antibodies conjugated to either FITC, Rhodamine Red or Cy 5 (Jackson ImmunoResearch Laboratories) were used for the co-visualisation of α-, β- and δ-cells within the islets (Leica Microsystems, TCS SP2). Cross-sectional islet areas and total pancreatic section areas were determined in H&E sections under light microscopy using image analyses software (AxioVision 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. 8 μm sections were cut and initially blocked with normal goat serum (10 % v/v) for 2 h, then incubated with rabbit anti-cleaved-caspased-3 (Cell Signalling) followed by detection using goat anti-rabbit Rhodamine red-X-conjugated secondary antibody (Jackson ImmunoResearch). Stained sections were viewed and photographed using inverted-phase fluorescence microscopy (Nikon).

**Transmission Electron Microscopy (TEM).** Pancreases were cut into 1mm³ pieces and fixed in 2% glutaraldehyde/0.1M Sorenson's phosphate buffer (pH 7.2, 4°C, overnight). Specimens were post-fixed with 0.1% osmium tetroxide/0.1M Sorenson's phosphate buffer (pH 7.2, 1 hour), 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 hour, room temperature) followed by 100% epoxy resin infiltration overnight. Specimens were then embedded with fresh resin and cured (48 hours, 60°C). Ultrathin sections were collected onto 200-mesh copper grids, double stained with 2% aqueous uranyl acetate (w/v) followed by lead citrate, prior to viewing (Phillips, CM-12).

**Statistical Analyses.** All data are expressed as mean ± SEM. Independent sample t-tests were performed using SPSS v11.5. 2-way ANOVA, General Linear Model and post-hoc Tukey’s HSD analyses were performed using Statistica 6.0. P values < 0.05 were accepted as statistically significant.
RESULTS

Over-expression 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 glucose tolerance tests (i.p.GTT) performed in fasted animals at the mid-diabetic stage (Fig. 2A) demonstrated impaired glucose tolerance and elevated fasting blood glucose (Fig. 2A) in human amylin transgenic mice at this age \((P < 0.05)\). The lack of glucose-stimulated insulin secretion during GTT present in human amylin transgenic mice \((t_0, 29.9 \pm 15.1\text{pM} \text{ cf. } t_{15}, 39.1 \pm 18.5\text{pM}, P = \text{ns})\), is consistent with impaired \(\beta\)-cell function. In contrast, we detected a positive insulin response to glucose in the control non-transgenic mice \((t_0 26.2 \pm 4.3\text{pM} \text{ cf. } t_{15} 128.6 \pm 55.7\text{ pM}, P < 0.05)\). Neither fasting hyperglycemia nor impaired glucose tolerance were present in \([25,28,29\text{Pro}]\)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 (i.p.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 non-transgenic 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 over-expression leads to decreased islet function. Plasma and pancreatic hormone levels were determined at the pre-diabetic, mid-diabetic and end-diabetic phases and equivalent times in non-diabetic 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 \(\beta\)-cell hormones: insulin \((P < 0.01)\) and total amylin \((P < 0.001)\). By contrast, in \([25,28,29\text{Pro}]\)human amylin transgenic mice, which did not develop diabetes, the pancreatic total amylin as well as insulin levels were significantly higher than in non-diabetic animals at all three phases (Table 1). This suggests that over-expression of human amylin results in \(\beta\)-cell destruction and impairment of \(\beta\)-cell function, which is consistent with our histological evidence of \(\beta\)-cell loss during diabetes progression (Fig. 4F). The decrease in \(\beta\)-cell mass in human amylin transgenic mice (Fig. 4H) confirms that the loss of \(\beta\)-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 ($P < 0.05$) whereas, 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, due to insufficient sample volumes.

The specificity of the amylin assay systems employed 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 the human amylin ELISA systems were validated to confirm that antibodies in each recognised the appropriate amylin species. Our validation experiments demonstrated that antibodies used in the ELISA specifically detected human amylin and did not cross-react with mouse amylin (Fig. 3A). Antibodies used in the RIA system were 100% cross-reactive with human amylin, murine amylin and $^{[25,28,29]}$Pro]human amylin, thus recognising human and murine amylin at equimolar concentrations but detecting $^{[25,28,29]}$Pro]human amylin at lower sensitivity compared to the former (Fig. 3B). Antibodies in both assay systems 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, over-expression 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.** 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. 4F). 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 non-diabetic 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 5J). About 60 islets from each study group (20 islets per animal and three animals per group) were examined and the total number of cleaved-caspase-3 positive cells determined. Numbers of apoptotic islet cells were found to be significantly increased in the TG mice at the mid-diabetic stage (Fig. 5I) compared to matched non-diabetic NT 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. 4G). 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 polarised light of Congo red-stained sections revealed the absence of green birefringence at all 3 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 itself is not necessary for and, indeed, 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 characterisation 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 developed diabetes (sustained hyperglycemia, Fig. 1A) and ~ 95% were diabetic by 22.5 weeks of age; by contrast, corresponding rates of diabetes in non-transgenic littermates were 0% (Fig. 1). Diabetes in L13 animals is associated with decreased \( \beta \)-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 non-transgenic mice at the mid-diabetic stage (Table 2), a 4-fold decrease in pancreatic insulin was present in transgenic mice during this period (Table 1). At the end-diabetic stage however, more than 17-fold decreases in pancreatic and plasma insulin were present (Tables 1 and 2). The progressive development of \( \beta \)-cell dysfunction over the diabetic process was paralleled by a progressive loss of insulin immunoreactive cells between the mid- (Fig. 4D) and end-diabetic stages (Fig. 4F) in transgenic islets, indicating progressive falls in the number of functional \( \beta \)-cells in hemizygous L13 mice. The disappearance of islet \( \beta \)-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 non-specific causation of diabetes by over-expression of protein within islet \( \beta \)-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 \( \beta \)-cells do not develop either islet \( \beta \)-cell disappearance or insulin-deficient diabetes (Fig. 4G), demonstrating that the insertion of this transgene and its expression in islet \( \beta \)-cells of a non-fibrillogenic amylin variant, is not in itself sufficient to cause \( \beta \)-cell destruction. Given that \( ^{25,28,29}\)Pro]human amylin mice expressing a non-aggregating amylin variant, do not develop diabetes (Fig. 2A) or islet \( \beta \)-cell degeneration (Fig. 4G), findings presented here support the idea that the development of diabetes in mice expressing wild-type human amylin (L13) are caused specifically by the expression of a fibrillogenic amylin variant in islet \( \beta \)-cells, rather than simply a non-specific event caused by over-expression 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 \( \beta \)-cell disappearance and diabetes, just as it is for its \textit{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 (1996), with which they are closely consistent, and 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 (1996) 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, is 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 (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-recognised. For example, Haluzik et al (2005) 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 occur in vitro (29-31). Here, we have shown that caspase-3 activation also occurs in pancreatic islets of L13 hemizygous mice but not by contrast in the islets of their non-transgenic littermates, both findings consistent with β-cell destruction through such an apoptotic pathway in these transgenic mice. Although both non-beta and beta 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 beta cells because of the known beta-cell targeting properties of the human amylin construct employed herein and the demonstrated loss of beta-cells from the islets of diabetic transgenic animals, whereas by contrast non-beta cells (glucagon- and somatostatin-containing) were relatively increased in the islets of late-diabetic animals (Fig. 4F). In addition, 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 over-expression 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 can not exclude the possibility of the presence of small human amylin aggregates/oligomers which 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 2-fold 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. Westermark 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 to hA+/+/mA+/+ mice was reportedly interpreted as consistent with an inhibitory effect of mouse amylin on fibril formation (33).

In summary, islet-specific over-expression 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. Over-expression 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 non-specific 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 comprise 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.

NON-STANDARD ABBREVIATIONS
ELISA, enzyme-linked immunosorbent assay; GTT, glucose tolerance test; ITT, insulin tolerance test; RIA, radioimmunoassay; TEM, transmission electron microscopy; TG, transgenic; NT, non-transgenic

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<tr>
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<td>2509.4 ± 775.3***/+++</td>
<td>2675.3 ± 103.7***/+++</td>
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<td><strong>Glucagon</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT</td>
<td>33.8 ± 3.4</td>
<td>27.4 ± 3.7</td>
<td>22.6 ± 1.7</td>
</tr>
<tr>
<td>TG</td>
<td>38.5 ± 6.0</td>
<td>27.9 ± 3.4</td>
<td>32.4 ± 4.0*</td>
</tr>
<tr>
<td><strong>Somatostatin</strong></td>
<td></td>
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</tr>
<tr>
<td>NT</td>
<td>5.2 ± 0.9</td>
<td>8.0 ± 0.7</td>
<td>6.4 ± 0.4</td>
</tr>
<tr>
<td>TG</td>
<td>4.7 ± 0.9</td>
<td>6.9 ± 1.0</td>
<td>6.5 ± 0.9</td>
</tr>
</tbody>
</table>

Hormones measurements were normalised to total pancreatic protein prior to statistical analyses. Data are expressed as mean ±SEM. *p<0.05, **p<0.01 and ***p<0.001; vs. NT; ++p<0.01 and +++p<0.001 vs. TG; n = 10.
**TABLE 2**
Plasma hormone levels (pM) at the pre-, mid- and end-diabetic stages in human amylin transgenic (TG) and non-transgenic (NT) animals.

<table>
<thead>
<tr>
<th>Stage of diabetes</th>
<th>Pre-diabetic</th>
<th>Mid-diabetic</th>
<th>End-diabetic</th>
</tr>
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<tbody>
<tr>
<td><strong>Insulin</strong></td>
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<tr>
<td>NT</td>
<td>120.5 ± 40.4</td>
<td>176.2 ± 36.8</td>
<td>178.4 ± 46.9</td>
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<tr>
<td>TG</td>
<td>78.3 ± 21.9</td>
<td>104.2 ± 31.5</td>
<td>10.0 ± 5.3*</td>
</tr>
<tr>
<td><strong>Human amylin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT</td>
<td>0.7 ± 0.4</td>
<td>1.2 ± 0.2</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>TG</td>
<td>27.7 ± 7.9**</td>
<td>14.9 ± 3.8*</td>
<td>3.7 ± 0.01*</td>
</tr>
<tr>
<td><strong>Glucagon</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT</td>
<td>22.6 ± 3.1</td>
<td>14.0 ± 4.3</td>
<td>11.9 ± 1.5</td>
</tr>
<tr>
<td>TG</td>
<td>33.0 ± 7.2</td>
<td>15.6 ± 3.5</td>
<td>43.0 ± 5.1***</td>
</tr>
</tbody>
</table>

Data are expressed as mean ±SEM. *p*<0.05, **p*<0.01 and ***p*<0.001; NT vs. TG; *n* = 10.
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 (open circles, n = 51), matched non-transgenic controls (closed circles, n = 49) and [25,28,29Pro]human amylin transgenic (closed squares, n=4). (B) Frequency of diabetes occurrence in the n = 51 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.
FIG. 2. Glucose tolerance, insulin tolerance and muscle glucose uptake during diabetes progression in human amylin transgenic mice. (A) i.p.GTT in human amylin transgenic (open circles, \(n = 18\)), [\(^{25,28,29}\)Pro]human amylin transgenic (open squares, \(n = 4\)) and non-transgenic control (closed circles, \(n = 16\)) mice. (B) i.p.ITT in human amylin transgenic (open circles, \(n = 15\)) and non-transgenic littermates (closed circles, \(n = 14\)). (C) *In vitro* glucose uptake in soleus muscles incubated without (basal) and with insulin (2mU/ml) in fasted human amylin transgenic (black bars, \(n = 7\)) and non-transgenic controls (white bars, \(n = 7\)). *\(P<0.05\), ** \(P<0.01\) and *** \(P<0.001\)
FIG. 3. Antibody cross-reactivity against human amylin and murine amylin measured in ELISA and RIA assay systems. (A) ELISA and (B) RIA; human amylin (closed circles), murine amylin (open squares), \[^{25,28,29}\text{Pro}]\text{human amylin} (closed squares) and human insulin (open circles).
FIG. 4. Expression patterns of insulin, glucagon and somatostatin, and quantitative histomorphometric analysis in pancreatic islets from transgenic and non-transgenic mice. Representative sections of insulin (green), glucagon (red) and somatostatin (cyan) immunolocalization in (A, C, E) non-transgenic control and (B, D, F) human amylin transgenic mice at (A, B) pre, (C, D) middle, and (E, F) late-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 non-transgenic 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 weights 1g. Results are expressed as fold changes of beta cell mass as relative to NT mice at Pre-diabetic stage, whose numbers were set at unity. +++ $P < 0.001$; NT Mid-diabetic/End-diabetic vs. NT Pre-diabetic. ***$P < 0.001$ TG vs. NT.
FIG. 5. Light and electron microscopic analysis of structure, and immunofluorescence (IF) study of apoptosis were performed in islets from representative non-transgenic and human amylin transgenic mice. Congo red-stained section imaged under polarised light of representative islets from (A, C, E) non-transgenic, and (B, D, F) transgenic mice at (A, B) pre-, (C, D) middle and (E, F) late stages. Birefringence is collagenous. Magnification = 400X. TEM images of islets from late-stage (G) non-transgenic and (H) human amylin transgenic mice. (G) Illustrates 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 late-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. (N), nucleus; (Ea), endocrine acini. Scale bars = 2 μm. IF staining for cleaved-caspase-3 in non-diabetic NT mice (I) and mid-diabetic TG 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 TG mice as compared to NT mice, whose numbers were set at unity. ***P < 0.001 versus NT.