Islet amyloid occurs in >90% of type 2 diabetic patients and may play a role in the pathogenesis of this disease. To determine whether islet amyloid occurs diffusely throughout the pancreas, whether it affects islets equally, and whether it decreases islet endocrine cells, we characterized islet amyloidosis by computerized fluorescence microscopy in transgenic mice that develop typical islet amyloid. These mice produce the unique amyloidogenic component of human islet amyloid, human islet amyloid polypeptide (hIAPP). The prevalence of amyloid (number of islets containing amyloid/total number of islets × 100) and the severity of amyloid (2amyloid area/2islet area × 100) were found to be uniform throughout the pancreas. Furthermore, a high prevalence of amyloid was observed in islets when the severity of amyloid was only 1.5% of the islet area, suggesting a diffuse distribution of amyloid from the very early stages of islet amyloidosis. In 12 hIAPP transgenic mice with an amyloid severity of 9.6 ± 3.4%, the proportion of islets composed of β- and δ-cells was reduced in the transgenic mice compared with 6 nontransgenic mice that do not develop amyloid (β-cells: 62.9 ± 3.1% vs. 75.5 ± 0.9%, P = 0.02; δ-cells: 2.8 ± 0.5% vs. 4.4 ± 0.4%, P = 0.05), whereas the proportion of islets composed of α-cells did not significantly differ between the two groups of mice. In the individual islets in these transgenic mice, amyloid severity was inversely correlated with β-cell, (r = −0.59, P < 0.0001), α-cell (r = −0.32, P < 0.0001), and δ-cell (r = −0.25, P < 0.0001) areas. In conclusion, islet amyloidosis occurs uniformly throughout the pancreas, affecting all islets before becoming severe. A reduction in islet endocrine mass starts at this early stage of islet amyloid development and progresses as amyloid mass increases.

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From the Division of Metabolism, Endocrinology and Nutrition, Department of Medicine, Veterans Affairs Puget Sound Health Care System and University of Washington, Seattle, Washington.

Address correspondence and reprint requests to Steven E. Kahn, M.B., Ch.B., Veterans Affairs Puget Sound Health Care System (151), 1660 S. Columbian Way, Seattle, WA 98108. E-mail: skahn@u.washington.edu.

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AUCglucose, area under the curve of glucose; AUCinsulin, area under the curve of insulin; CCD, charge-coupled device; CV, coefficient of variation; Cy3, indocarbocyanine; hIAPP, human islet amyloid polypeptide; IAPP, islet amyloid polypeptide; IPGTT, intraperitoneal glucose tolerance test.
In the present study, we developed a method to precisely quantify islet amyloid and cellular components. Using this method, we addressed the following questions in our hIAPP transgenic mice: 1) How is islet amyloid distributed in different pancreatic regions? 2) Is the prevalence of amyloid in all islets related to the amount of amyloid in individual islets? and 3) Does the endocrine cell composition change in islets containing moderate amounts of amyloid?

RESEARCH DESIGN AND METHODS

hIAPP transgenic mice. Mice expressing the hIAPP gene in their pancreatic \( \beta \)-cells were bred at the University of Washington (13). By breeding male hemizygous hIAPP transgenic mice (C57BL/6xDBA/2) with female nontransgenic mice (C57BL/6xDBA/2; Jackson Laboratories, Bar Harbor, ME), we produced offspring that did or did not express the hIAPP transgene. Genotyping was performed by polymerase chain reaction using primers specific for hIAPP (18). Because islet amyloid occurs more frequently in male than in female hIAPP transgenic mice (16), only male mice were studied. Male nontransgenic littermates were used when control mice were necessary. All of the studies were approved by the Animal Care Committee at the VA Puget Sound Health Care System.

Preparation of pancreatic sections. To investigate the distribution of islet amyloid, the pancreases, designated as pancreases A, B, and C, were obtained from hIAPP transgenic mice. These mice were 15 months old and fed a diet containing 9% fat wt/wt (Mouse Diet 5021; Purina Mills, St. Louis, MO). Each pancreas was fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.2) and embedded in paraffin with the pancreatic head oriented toward the top of the paraffin block. Each pancreas was cut completely, yielding 2,700–3,500 sections (5 \( \mu \)m thick). We selected 20 sections at a fixed interval (\( \sim 750 \mu \)m) throughout each pancreas. The sections were stained with thioflavin-S to visualize islet amyloid.

To determine whether the distribution of \( \beta \)-cells in the pancreas differs between hIAPP transgenic and nontransgenic mice, sections were taken from the head, body, and tail of three hIAPP transgenic pancreases (pancreases A, B, and C) and three nontransgenic pancreases (pancreases D, E, and F), the latter obtained from mice of the same age and genetic background. None of the six mice had hyperglycemic. The sections were stained by immunofluorescence to visualize the portion of the islet composed of insulin-positive cells.

The method for thioflavin-S staining has been described previously (16). To visualize islet endocrine cells, pancreatic sections were incubated for 18 h with an antiserum against either insulin (1:2,000, Sigma I2018; Sigma, St. Louis, MO), glucagon (1:20,000, 14C; a gift from Dr. Robert McEvoy), or somatostatin (1:1,000, AS10; a gift from Dr. John Emsinck). The sections were rinsed with phosphate buffer and incubated for 1 h with secondary antisera (1:250; Jackson ImmunoResearch, West Grove, PA) conjugated with indocarbocyanine (Cy3). The method for immunofluorescence staining has been described elsewhere (19).

Computerized fluorescence microscopy. A fluorescence microscope (Axio-iplan; Zeiss, Gottingen, Germany) was equipped with a charge-coupled device (CCD) camera operating in 10 bits (Hamamatsu C4880; Photonics, Hamamatsu City, Japan). The computer-based imaging system was supported by MCID-M2 software (Image Research; St. Catherines, ON, Canada). When stained with thioflavin-S, islet amyloid fluoresced green at E, 480 nm and E\( _{\text{exc}} \), 505 nm. When stained with Cy3, islet areas positive for either insulin, glucagon, or somatostatin fluoresced orange at E, 535 nm and E\( _{\text{exc}} \), 575 nm. With either thioflavin-S or Cy3 staining, pancreatic islets were clearly differentiated from exocrine tissue (16).

Iset amyloid area stained by thioflavin-S or Cy3 was quantified as follows. The islet was located, and the target area was visualized using the appropriate filter. The islet image was acquired by the CCD camera and projected onto a computer screen. Density thresholds were selected using a slider control in the dialog box. In this process, islet areas having optical densities in the selected range were highlighted in a pseudocolor. The thresholds were set when the highlighted area coincided with the fluorescent area in the microscope. The area stained by fluorescence was thereby defined as the target. The islet was outlined with a click-outline tool to define the area to be scanned. The islet was then auto-scanned, and both target and total islet areas were calculated by the computer. The coefficient of variation (CV) for the measurements performed by investigators blinded to the nature of the specimens was 5% and the same investigator and 7–10% between investigators.

When the distribution of amyloid and \( \beta \)-cells was studied, all islets in each pancreatic section were examined. When islet cell composition was studied, three consecutive sections of each pancreas were first previewed. Islets that were identified in all three sections were included for further evaluation. In each pancreas, ~10% of the islets were found in only one or two sections and were therefore excluded from the study. After the preview, islets were measured individually. In each section, the three images with staining for insulin, glucagon, and somatostatin were acquired from the consecutive sections, whereas the image with staining for amyloid was obtained from the second section. After all images were displayed in separate channels on the computer screen, target and total islet areas were measured channel by channel as described above. The validity of quantifying amyloid in one section was verified in a preliminary study in which the amyloid areas in 27 islets were quantified in all three consecutive sections. Both amyloid area and total islet area were consistent among the consecutive sections, with a CV of 4% for the islet amyloid fraction.

Computation of microscopic results. Two measures were used to describe the degree of amyloidosis in all islets examined in a pancreatic section. Amyloid prevalence denoted the frequency of islets containing amyloid (number of islets containing amyloid/total number of islets). Whereas amyloid severity denoted the proportion of total islet area occupied by amyloid deposits (\( \text{Amyloid area/2islet area} \times 100 \)). To calculate islet cell composition in a mouse, the area positive for either insulin, glucagon, or somatostatin was summed up in all islets examined and divided by total islet area (\( \text{2islet area/2islet area} \times 100 \)). The results were used to represent the proportion of the islet composed of \( \beta \)-, \( \alpha \)-, or \( \delta \)-cells in the given mouse. Based on these results, islet proportions occupied by these endocrine cells were calculated in each mouse group. The mean islet size in a group was calculated from the mean islet size of each individual mouse in the group.

Plasma analyses. The 12 hIAPP mice and 6 nontransgenic littersmates included in the study of islet cell composition underwent an intraperitoneal glucose tolerance test (IPGTT) 1 week before they were sacrificed. The mice were anesthetized (pentobarbital 100 mg/kg i.p.) after an overnight fast. Blood was sampled from the retroorbital sinus before and 15, 30, 60, 120 min after the glucose load (1 g/kg i.p.). Plasma glucose concentrations were determined by the glucose oxidase method, and insulin concentrations were measured by radioimmunoassay (20). Immediately before they were sacrificed, blood was sampled again after a 4-h fast, and plasma hIAPP was determined using an enzyme-linked immunosorbent assay (20).

Statistics. Data are presented as the means ± SE. The area under the curve for glucose and insulin during the IPGTT was calculated using the trapezoidal approach. The Kruskal-Wallis test was used for multiple comparisons or when only two groups were compared. The Pearson correlation was used to examine the relationship between two parameters. \( P \leq 0.05 \) was considered significant.

RESULTS

Quantification of islet amyloid. All amyloid deposits visible at magnifications of 100\( \times \) and 200\( \times \) were quantified by the image analysis system. At 200\( \times \), the smallest amyloid deposit occupied one pixel (1.44 \( \mu \)m\(^2\)). In the 15 hIAPP transgenic mice examined in this study, the mean islet area was 51,800 ± 6,500 \( \mu \)m\(^2\), and the smallest islet area was 800 \( \mu \)m\(^2\). Thus, the single-pixel amyloid accounted for <0.01% and <1% of the area of average-sized and small islets, respectively. No islet amyloid was observed in the pancreases of nontransgenic mice.

Iset amyloidosis occurs consistently throughout the pancreas. In the 20 sections from each of the mice examined for the distribution of islet amyloid, the number of islets per section was similar (5.9 ± 0.9, 9.1 ± 1.3, and 7.3 ± 1.5 for pancreases A, B, and C, respectively.)
Within each pancreas, amyloid severity and prevalence were largely uniform in islets found in each of the 20 sections (Fig. 1). When the sections (n = 20) of each pancreas were divided into three groups representing the head (n = 6), body (n = 7), and tail (n = 7) of the pancreas, the mean amyloid prevalence and severity were uniform among the three anatomical regions (Table 1). This uniform distribution occurred within each pancreas, even though the amyloid severity and prevalence varied among the three pancreases (Table 1). Furthermore, all three pancreases had a high prevalence of amyloid (≥85%), although the severity of amyloid in the pancreases varied from 1.5 to 40%. This finding suggested that a high prevalence of amyloid was attained when only small amyloid deposits were formed in individual islets.

**Islet amyloid involves nearly all islets at an early stage.** To determine whether a high prevalence of islet amyloid was achieved even when smaller amounts of amyloid were present, we investigated the relation between amyloid prevalence and amyloid severity in the three pancreases described above and in another 12 hIAPP transgenic pancreases that were prepared for the study of islet cell composition. As described in the RESEARCH DESIGN AND METHODS section, the 12 additional pancreases were embedded so as to contain two or three random specimens of a single pancreas in a section. In view of our observation that amyloid deposition was uniform in the pancreas, 20 islets on one section were examined in each of the 12 additional pancreases. As shown in Fig. 2, amyloid severity and prevalence from the 15 mice were positively and nonlinearly correlated. After log transformation, the equation between these two parameters was \[ \log_2(\text{severity}) = 5.15 \times \log_2(\text{prevalence}) - 21.16 \] (r = 0.78, P = 0.001). A high amyloid prevalence (≥80%) was found when total amyloid area was equal to 1.5% of the total islet area, a finding similar to that in the three hIAPP transgenic pancreases. In some sections we did observe variability in the severity of amyloidosis, with some islets...
containing more amyloid than others. However, in no pancreas did we observe severe islet amyloidosis without a high prevalence of islet involvement.

**Contribution of β-cells in islets is consistent within a mouse pancreas.** Table 2 shows the contribution of β-cells in islets located in the head, body, and tail of the pancreases from either hIAPP transgenic or nontransgenic mice. The β-cell contribution was consistent within each pancreas. In addition, the β-cell contribution was comparable among the three nontransgenic pancreases. In the three hIAPP transgenic pancreases, the β-cell contribution differed (pancreas A>B>C), and this difference appeared to be inversely related to the differences in amyloid severity observed in the same pancreases (pancreas A<B=C) (Table 1).

**Moderate amounts of amyloid alter islet endocrine cell composition.** Islet cell composition was studied in 12 hIAPP transgenic mice, using 6 nontransgenic littermates as controls. The number of islets examined per mouse was 20 ± 2 (range 12–30) in hIAPP mice and 20 ± 5 (13–40) in the controls. The mean islet size was 45,700 ± 7,000 μm² in hIAPP mice and 50,700 ± 9,000 μm² in controls; this difference was not significantly different. All but one hIAPP mouse had islet amyloid, and the mean amyloid severity was 9.6 ± 3.4% (n = 12). As expected, none of the nontransgenic mice had islet amyloid.

Table 3 shows the proportion of islet area positive for insulin, glucagon, or somatostatin in hIAPP transgenic and nontransgenic mice. These data represent the contribution of β-, α-, or δ-cells in the islets, respectively. In hIAPP mice, the proportion of islets composed of β- and δ-cells was significantly decreased compared with the controls. No significant difference was found between the transgenic and nontransgenic mice in the proportion of islets occupied by α-cells. In both groups of mice, a large proportion of islet area (~15%) was comprised of vascular cells and space.

The effect of amyloid on islet cell composition was also assessed in individual islets (n = 240) examined in the 12 hIAPP mice. Amyloid severity was inversely correlated with β-cell (r = −0.59, P < 0.0001), α-cell (r = −0.32, P < 0.0001), and δ-cell (r = −0.25, P < 0.0001) areas, as shown in Fig. 3A, B, and C, respectively.

**Glucose metabolism in mice with moderate amounts of islet amyloid.** At the time they were sacrificed, the 12 hIAPP mice and 6 nontransgenic mice had similar body weights (64 ± 3 vs. 61 ± 4 g). The plasma hIAPP concentration was 80 ± 19 pmol/l in hIAPP mice (n = 11) and undetectable in nontransgenic mice. Table 4 shows the results of the IPGTT performed 1 week before the mice were sacrificed. Fasting glucose and insulin were not significantly different between hIAPP and nontransgenic controls.

### Table 1

<table>
<thead>
<tr>
<th>Mice</th>
<th>Head</th>
<th>Body</th>
<th>Tail</th>
<th>Overall</th>
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<tbody>
<tr>
<td>Amyloid severity (%)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Pancreas A</td>
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<td>2.2 ± 0.6 †</td>
<td>1.5 ± 0.3 †</td>
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<tr>
<td>Pancreas B</td>
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<td>19 ± 1 †</td>
<td>14 ± 2 †</td>
<td>17 ± 1 †</td>
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<tr>
<td>Pancreas C</td>
<td>39 ± 2 †</td>
<td>46 ± 4 †</td>
<td>36 ± 4 †</td>
<td>40 ± 2 †</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.01, †P < 0.001, and ‡P < 0.0001 compared to the other two pancreases.

### Table 2

| Contribution of β-cells in islets located in different pancreatic regions |
|-----------------------------|-----------------------------|-----------------------------|
| Mice                        | Head | Body | Tail | Overall |
| hIAPP transgenic            |      |      |      |         |
| Pancreas A                  | 70 ± 3 (9) † | 70 ± 2 (12) † | 69 ± 2 (9) † | 69 ± 1 (30) † |
| Pancreas B                  | 56 ± 3 (12) † | 55 ± 3 (15) † | 55 ± 3 (11) † | 56 ± 2 (38) † |
| Pancreas C                  | 24 ± 2 (8) † | 31 ± 1 (6) † | 31 ± 2 (22) † | 30 ± 1 (36) † |
| Nontransgenic               |      |      |      |         |
| Pancreas D                  | 76 ± 2 (8) | 72 ± 3 (8) | 77 ± 3 (9) | 75 ± 2 (25) |
| Pancreas E                  | 77 ± 1 (4) | 75 ± 4 (5) | 72 ± 3 (6) | 74 ± 2 (15) |
| Pancreas F                  | 76 ± 3 (8) | 74 ± 2 (7) | 76 ± 3 (9) | 76 ± 1 (24) |

Data are means ± SE (n), with n = number of islets examined. *P < 0.001 and †P < 0.0001 compared with the other two transgenic pancreases.

### Table 3

| Endocrine cell composition of islets in 12 hIAPP transgenic and 6 nontransgenic mice |
|---------------------------------------------|-----------------------------|-----------------------------|
| Mice                        | Islet component (% of total islet area) |
|                            | Insulin area  | Glucagon area | Somatostatin area |
| hIAPP transgenic           | 62.9 ± 3.1 † | 7.7 ± 1.3 | 2.8 ± 0.5 † |
| Nontransgenic              | 75.5 ± 0.9 | 6.3 ± 0.4 | 4.4 ± 0.4 |

Data are means ± SE. *P = 0.02 and †P = 0.05 compared with nontransgenic controls.

FIG. 2. Relation between amyloid severity and amyloid prevalence. Amyloid severity (amyloid area/islet area × 100) and amyloid prevalence (no. of islets containing amyloid/no. of islets × 100) were nonlinearly related in 15 hIAPP transgenic mice. Amyloid prevalence increased, so that the vast majority of islets were involved before amyloid severity increased.
significantly different between hIAPP mice and controls. The cumulative response of glucose, calculated as the area under the curve of glucose (AUC$_{\text{glucose}}$), tended to be increased in hIAPP mice compared with controls. The cumulative response of insulin, calculated as the area under the curve of insulin (AUC$_{\text{insulin}}$), was significantly decreased in transgenic mice compared with controls. Hence, the ratio of AUC$_{\text{insulin}}$ to AUC$_{\text{glucose}}$ was significantly decreased in hIAPP mice compared with control mice.

DISCUSSION

In this study, amyloid prevalence and amyloid severity were found to be consistent in 20 islet samples taken uniformly throughout the pancreas of hIAPP transgenic mice. To our knowledge, this is the first report on the distribution of islet amyloid in entire pancreases using the present sampling approach. This observation demonstrates that islet amyloid is distributed in a diffuse and uniform fashion throughout the pancreas in hIAPP mice. Based on this observation, islet amyloidosis found on a single pancreatic sample is likely to represent the overall state of islet amyloidosis in the pancreas, although the approach of using the average result from 2–3 samples from different parts of the pancreas may minimize the random variation among sections.

In the present study, hIAPP transgenic mice that were of the same age and fed the same diet showed different degrees of islet amyloidosis. Likewise, degrees of islet amyloidosis have been shown to vary in type 2 diabetic patients (21). These observations suggest that islet amyloidosis may start at different ages and/or progress at different rates. However, we did find in this study that the severity of islet amyloid was related to the prevalence of islet amyloid in a nonlinear manner, thus suggesting that islet amyloidosis develops through a common pathway that involves two phases. The first phase is characterized by a diffuse commencement of amyloid formation in the whole islet population, whereas the second phase is characterized by a progressive accumulation of amyloid in individual islets. Once amyloid deposition is ~1.5% of the total islet area, the transition from first to second phase appears to occur. A similar scenario likely occurs in human pancreas. Examination of the data of Westermark (21) suggests that in humans the transition from the first to second phase occurs when the amyloid area occupies ~5% of the total islet area, a point at which the majority of islets exhibit islet amyloid. Thus, in both human and hIAPP mouse pancreases, islet amyloid is diffusely distributed and is likely a manifestation of a change in islet function that affects all islets. Therefore, it is conceivable that any preventive measure taken at an early stage of islet amyloidosis may arrest the subsequent accumulation of amyloid in all islets.

We also found that the proportion of islets composed of β-cells was uniform throughout the pancreas in both hIAPP transgenic and nontransgenic mice. This uniformity in β-cell composition in both groups of mice is in keeping with the diffuse distribution of islet amyloid.
with the findings of Baetens et al. (22) in normal rats. Because hIAPP, the major constituent of islet amyloid, is secreted from islet β-cells, the consistent distribution of β-cells may underlie the consistent distribution of islet amyloid in the pancreas of hIAPP transgenic mice, even when amyloid severity is low. Likewise, the distribution of islet amyloid appears to coincide with the distribution of islet β-cells in human pancreas (23). Thus, the fact that the proportion of the islet composed of β-cells is lower in the pancreatic polypeptide (PP) cell–rich (and α-cell–poor) posterior part of the pancreatic head than in the remainder of the pancreas (23), islet amyloid may be more prevalent in other lobules of the human pancreas than in the posterior part of the pancreatic head.

In previous studies, islet amyloidosis, especially at its late stages, was associated with a decrease in the proportion of the islet composed of β-cells (5,6). In the present study, the proportion of the islet composed of β-cells was decreased in hIAPP mice with moderate islet amyloidosis compared with nontransgenic mice without islet amyloid. In a previous study of human pancreatic islets, decreased β-cell mass was found with a mean amyloid severity similar to that seen in the present study (24). Both the present and previous studies suggest that islet β-cell mass does decrease in islets with moderate amounts of amyloid. In addition, we found that islet δ-cell mass was also decreased in hIAPP mice compared with control mice. This finding supports the notion that non-β endocrine cells are also decreased in amyloid-containing islets (5). In a previous study in monkeys, when β-cell mass was first decreased in amyloid-containing islets, the proportion of islets composed of α-cells was relatively increased. However, as more amyloid was deposited, the α-cell proportion decreased (5). These sequential changes in the proportion of islets composed of α-cells may explain why the mean α-cell contribution in the 12 hIAPP mice with varying islet amyloid severity did not differ from that in 6 nontransgenic mice. When the effect of amyloid on islet endocrine cells was assessed in individual islets, amyloid mass was found to be inversely related to the proportions of the islet composed of β-, α-, and δ-cells. This observation suggests that the diminution of endocrine mass is a continuous process that is closely related to the increase of amyloid mass in islets.

In the present study, hIAPP mice with moderate islet amyloidosis demonstrated impaired β-cell function and a tendency toward reduced glucose tolerance compared with nontransgenic mice fed the same high-fat diet. However, it has been suggested that IAPP per se may influence glucose homeostasis (25,26). Thus, it is not clear to what extent the altered islet composition in our hIAPP transgenic mice can be implicated in the abnormal glucose tolerance and insulin secretion seen in this study. However, we have previously demonstrated using pancreas perfusion that in our hIAPP transgenic mice at 3 months of age, glucose-induced insulin release is increased compared with age-matched nontransgenic controls (27). Thus, the impairment in insulin release seen in the present study may be attributable in part to the difference in age and/or islet amyloidosis. It is not inconceivable that the effect of islet amyloid on insulin secretory capacity may increase further as islet amyloidosis progresses.

In summary, we have found that islet amyloid in the pancreas of hIAPP transgenic mice is diffuse and uniform, affecting all islets before becoming severe. The reduction of islet endocrine cells occurs at the early stages of islet amyloidosis and continues with the progression of amyloidosis. The diffuse and progressive nature of islet amyloid and the close relation of islet amyloid to islet endocrine mass underscores the potential importance of arresting islet amyloidosis at its early stages.

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