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Intra- and extracellular amyloid fibrils are formed in cultured pancreatic islets of transgenic mice expressing human islet amyloid polypeptide

(non-insulin-dependent diabetes mellitus)

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ABSTRACT **Islet amyloid polypeptide (IAPP) is the constituent peptide of amyloid deposits found in the islets of non-insulin-dependent diabetic patients. Formation of islet amyloid is associated with a progressive destruction of insulin-producing beta cells. Factors responsible for the conversion of IAPP into insoluble amyloid fibrils are unknown. Both the amino acid sequence of human IAPP (hIAPP) and hypersecretion of hIAPP have been implicated as factors for amyloid fibril formation in man. We have generated transgenic mice using rat insulin promoter–hIAPP or rat IAPP (rIAPP) gene constructs. No fibrillar islet amyloid was detectable *in vivo* in these normoglycemic mice, although small amorphous perivascular accumulations of IAPP were observed in hIAPP mice only. To determine the effects of glucose on IAPP secretion and fibrillogenesis, pancreatic islets from transgenic and control mice were examined *in vitro*. Islet IAPP secretion and content were increased in transgenic islets compared with control islets. IAPP-immunoreactive fibrils were formed at both intra- and extracellular sites in isolated hIAPP islets cultured with glucose at 11.1 and 28 mM for only 7 days. At 28 mM glucose, fibrils were present in deep invaginations of beta cells as observed in non-insulin-dependent diabetic patients. No fibrils were present at low glucose concentrations in hIAPP islets or at any glucose concentration in rIAPP or control islets. Thus, glucose-induced expression and secretion of hIAPP in transgenic mouse islets can lead to formation of amyloid fibrils similar to that found in non-insulin-dependent diabetes mellitus.**

Pancreatic islet amyloid, formed from islet amyloid polypeptide (IAPP) (1, 2), is found in up to 98% of patients with non-insulin-dependent diabetes mellitus (NIDDM) (3–5). This pathological change is associated with beta-cell destruction (6, 7). Furthermore, the localization of amyloid fibrils between islet cells and capillaries is likely to contribute to deterioration of islet function, which is characteristic of the disease (8, 9). The severity of islet amyloidosis in the pancreas of diabetic patients is greater in those patients who have progressed from sulphonylurea therapy to insulin treatment (9). The species-specific amino acid sequence of IAPP is a determining factor in conversion of IAPP into amyloid fibrils (10). IAPP-derived amyloid is found in association with diabetes in man, monkeys, cats, and raccoons but not in rodent models of diabetes (5, 11). Transgenic mice were generated expressing the gene for human IAPP (hIAPP) or rat IAPP (rIAPP), which is identical to mouse IAPP (mIAPP), under transcriptional control of a rat insulin 2 gene promoter sequence (12). Amyloid fibrils were not detected in islets from hIAPP transgenic mice up to 86 weeks old despite the presence of the hIAPP amino-acid sequence and elevation of circulating IAPP concentrations by a factor of 2–15 (12). However, small amorphous perivascular accumulations of IAPP were present between beta cells and capillaries in some hIAPP transgenic mice, indicating that hIAPP can accumulate at similar sites in islets of transgenic mice and of man (13). Furthermore, hIAPP and rIAPP transgenic mice were normoglycemic, normoinsulinemic and were not obese (12). This suggests that elevated plasma IAPP ("amylin") in these mice does not have a significant physiological role in inhibition of insulin action (14) or secretion (15).

In this transgenic mouse model, glucose will activate the rat insulin 2 gene promoter, which drives expression of the transgene, and will stimulate release of insulin and IAPP. To examine the effects of elevated glucose concentrations on fibrillogenesis, islets were isolated from hIAPP and rIAPP transgenic and nontransgenic mice and were incubated *in vitro* at various concentrations of glucose.

MATERIALS AND METHODS

Islet Isolation. Transgenic mice expressing the gene for hIAPP (eleven 16- to 42-week-old mice from three independent breeding lines) and for rIAPP (eleven 27- to 43-week-old mice from two independent breeding lines) and nontransgenic littermates (twelve 17- to 33-week-old control mice) were used in the studies. Transgene incorporation was confirmed by Southern blot analysis of chromosomal DNA extracted from tail biopsies as described (12). Anesthetized mice were killed, and the pancreas was removed. After collagenase digestion of the pancreas (16), islets were handpicked and maintained free-floating in tissue culture at 37°C in humidified air containing 5% CO_2. The culture medium was RPMI 1640 containing 10% (vol/vol) fetal calf serum, benzylpenicillin (100 units/ml), streptomycin (0.1 mg/ml), and initially 11.1 mM glucose.

Experimental protocols for physiological studies were started on day 7 after islet isolation. To determine the pattern of long-term insulin and IAPP secretion, 50 islets were maintained in tissue culture for 6 days with replacement of the medium every 48 hr. The mean was calculated from the

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Abbreviations: NIDDM, non-insulin-dependent diabetes mellitus; IAPP, islet amyloid polypeptide; hIAPP, human IAPP; rIAPP, rat IAPP; mIAPP, mouse IAPP.

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peptide concentration of three successive samples because there was no statistical difference in secretion over the 6-day period. Measurement of islet IAPP and insulin content and 1-hr insulin-release experiments at 1.7 and 16.7 mM glucose were performed as described (17). Samples were stored at −20°C.

Radioimmunoassay. Measurement of insulin was performed in duplicate by radioimmunoassay (Pharmacia RIA-100) against a rat insulin standard. IAPP concentrations were measured in duplicate by using a standard radioimmunoassay (Peninsula Laboratories). The antibody used in this assay cross-reacted equally with hIAPP, rIAPP, and mIAPP.

Electron Microscopy. After isolation, pancreatic islets were maintained for 7 days in RPMI 1640 tissue culture medium containing 3.3, 11.1, or 28 mM glucose and were subsequently fixed in 2% paraformaldehyde and 0.25% glutaraldehyde in phosphate buffer (pH 7.2). Specimens were postfixed in 1% osmium tetroxide, dehydrated, and embedded in an epoxy resin. Ultrathin sections were cut and mounted on nickel grids. Specimens were immunogold-labeled for IAPP by use of an antiserum against rIAPP (diluted 1:1000), which cross-reacts with hIAPP and mIAPP (Peninsula Laboratories), and for insulin by use of an antiserum to bovine insulin (diluted 1:1000) (ICN). Antisera binding sites were identified with protein A-conjugated gold (10 or 15 nm) (Biocell Laboratories). Specificity of the antisera was confirmed by loss of immunoreactivity after preabsorption with the appropriate antigen (0.01 mg/ml). Contrast of the sections was enhanced with uranyl acetate and lead citrate. At least five isolated islets per mouse were systematically examined for the presence of amyloid fibrils.

Statistical Analysis. Differences between hIAPP or rIAPP islets and control islets were analyzed by use of the Wilcoxon rank-sum test. Probability values of <0.05 were considered to be significant.

RESULTS AND DISCUSSION

IAPP secretion from transgenic islets and IAPP content of transgenic islets were significantly increased compared with that of islets from nontransgenic mice (Fig. 1 *A* and *B*). IAPP-immunoreactive fibrils were present between cells of hIAPP islets cultured at 11.1 mM glucose (Fig. 2). Fibril formation was particularly dramatic in hIAPP islets cultured at 28 mM glucose; fibrils appeared to stream into the extracellular space from deep invaginations of the beta-cell membrane (Fig. 3). Some cells were almost completely filled with amyloid fibrils (Fig. 4 *Left*). Similar irregularities in the beta-cell border adjacent to amyloid are found in islets of diabetic cats and man (8, 18, 19). In hIAPP islets cultured at 3.3 mM glucose, no extracellular fibrils were observed. Fibrils were never seen in rIAPP islets despite increased levels of secretion of IAPP or in control islets at any glucose concentration (Fig. 1*A*).

The formation of amyloid fibrils by hIAPP islets *in vitro* but not *in vivo* indicates that conditions that promote fibrillogenesis are present in tissue culture. Some of these conditions are likely to be similar to those present in islets of patients with NIDDM. The accumulation of fibrils between islet cells *in vitro* suggests that there is insufficient clearance of secreted hIAPP; concentrations of hIAPP high enough to induce fibrillogenesis (10) could be achieved between the cells of hIAPP islets. In NIDDM, amyloid fibrils are often deposited between beta cells and islet capillaries, suggesting that impaired clearance of IAPP from the extracellular space may be important. In addition, a role for other extracellular factors (e.g., glucose) in fibrillogenesis of hIAPP cannot be excluded. The absence of extracellular IAPP-immunoreactive material in rIAPP and control islets suggests that metabolism and clearance of rIAPP and mIAPP in isolated islets is undisturbed.

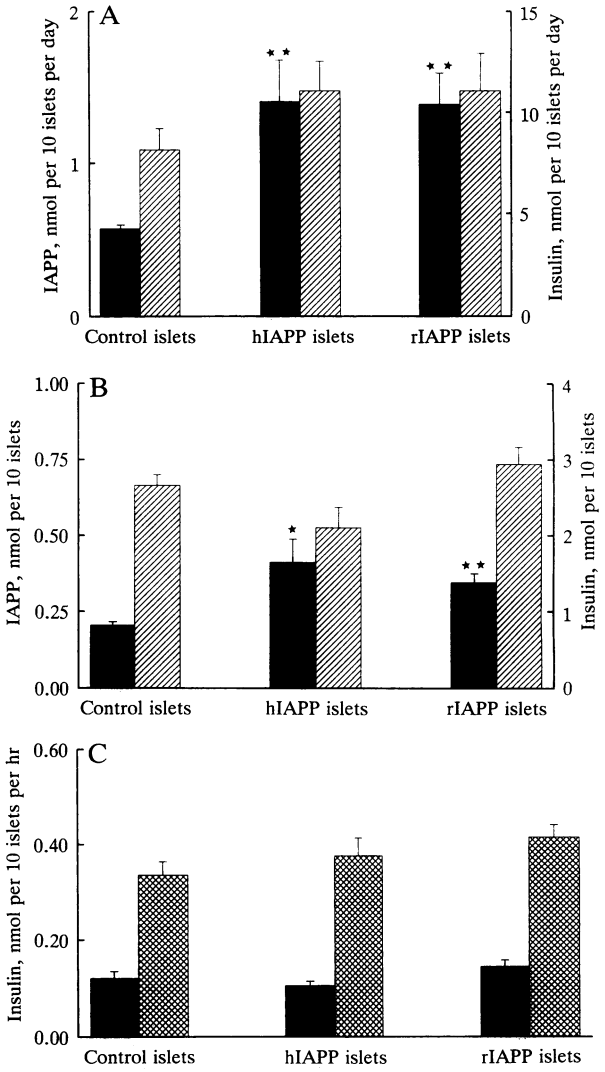

FIG. 1. (*A* and *B*) Secretion of insulin (▨) and IAPP (■) and peptide content of hIAPP, rIAPP, and control islets (control). (*A*) Concentrations of insulin and IAPP secreted into tissue culture medium during long-term incubation (nmol per 10 islets per day) in the presence of 11.1 mM glucose. Secretion of IAPP from hIAPP and rIAPP islets was higher than that from control islets ($P < 0.005$). (*B*) IAPP and insulin content of cultured islets (nmol per 10 islets). IAPP content was higher in hIAPP islets ($P < 0.05$) and rIAPP islets ($P < 0.005$) than in control islets. The molar ratio of IAPP:insulin secreted from hIAPP and rIAPP islets (1:8.5 and 1:7.9, respectively) was increased compared with control islets (1:15.1); a similar increase was present in the molar ratio of IAPP:insulin stored in the islets (1:6.3 and 1:9.0 versus 1:13.3). (*C*) Static short-term insulin secretion during successive 1-hr incubation periods in 1.7 (■) and 16.7 (▩) mM glucose (nmol per 10 islets per hr). The glucose-stimulated insulin secretion was not different in transgenic islets compared with control islets. Data are expressed as means ± SE with 9–12 observations for each of the three groups. *, $P < 0.05$; **, $P < 0.005$.

Fibrils immunoreactive for IAPP were also observed within beta-cell cytoplasm of hIAPP islets cultured at 11.1 mM glucose (Fig. 4 *Right*). These fibrils were of similar dimensions and appearance as the extracellular fibrils. Cytoplasmic fibrils have been observed in beta cells of human insulinomas (20), which hypersecrete beta-cell products. Insulin secretion and insulin content of islets were similar in transgenic and control islets (Fig. 1). Thus, the presence of increased concentrations of endogenous IAPP adjacent to

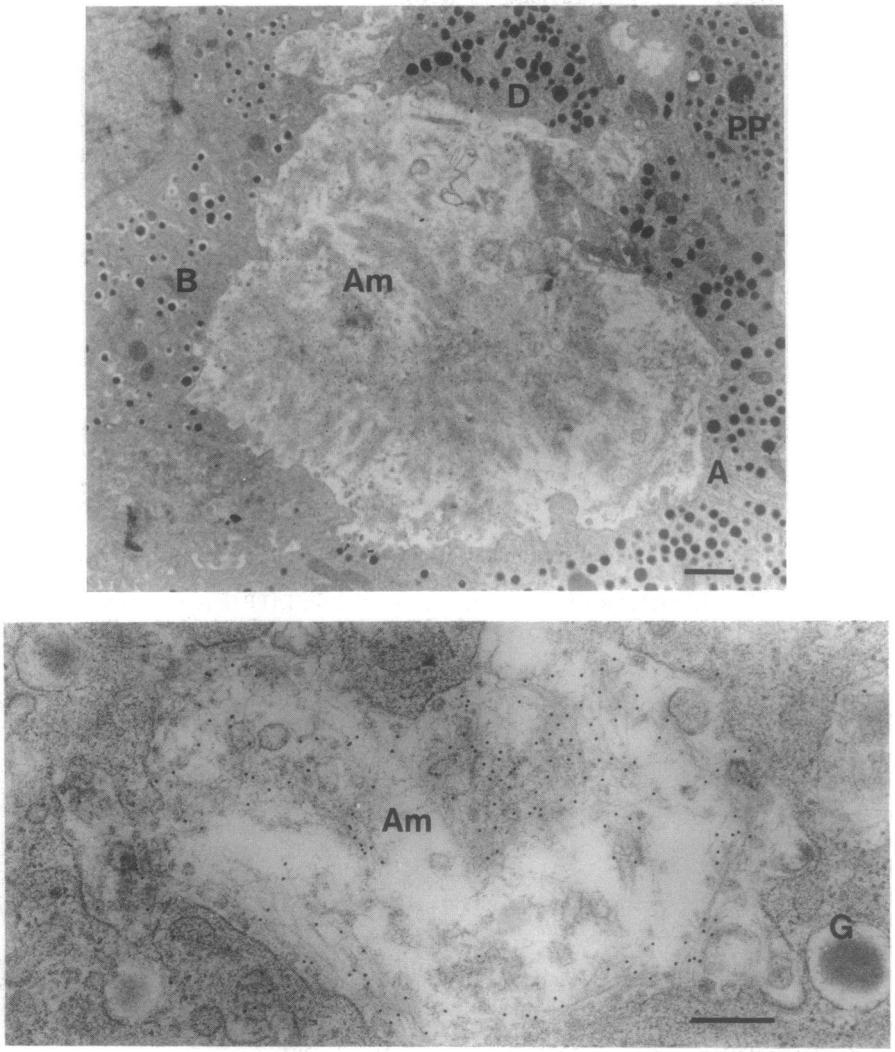

FIG. 2. Electron micrographs of pancreatic islets isolated from hIAPP transgenic mice maintained in tissue culture for 7 days at different glucose concentrations. (*Upper*) hIAPP islet cultured at 11.1 mM glucose. IAPP-immunoreactive fibrils (Am) are present in a large extracellular space surrounded by islet cells. B, insulin-containing beta cell; A, glucagon-containing alpha cell; PP, pancreatic polypeptide-containing PP cell; D, somatostatin-containing delta cell. Amyloid deposits were found in extracellular spaces distributed throughout the islets but always adjacent to one or more beta cells. The deposits were found in islets isolated from all three independent breeding lines of hIAPP transgenic mice. (Bar = 1 μm.) (*Lower*) Extracellular fibrils immunoreactive for IAPP in a hIAPP islet cultured at 11.1 mM glucose. Fibrils (Am) are irregularly distributed in a space between two beta cells. G, secretory granule. These fibrils were similar in size (8–10 nm in diameter) to amyloid fibrils in islets of subjects with NIDDM. No immunoreactivity for insulin was present on extracellular proteinaceous deposits in hIAPP islets. (Bar = 0.25 μm.)

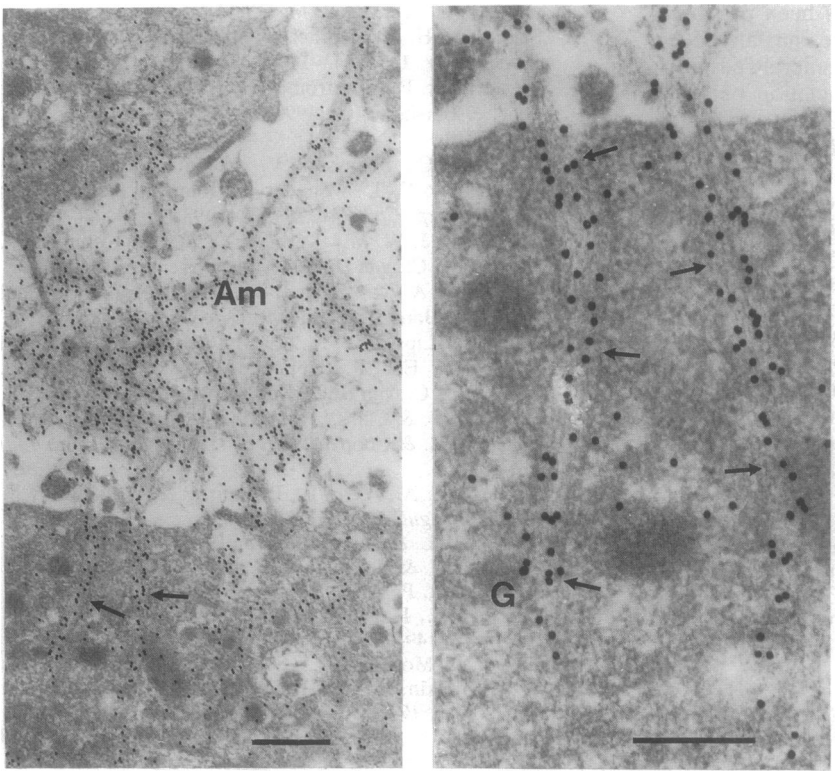

FIG. 3. Extracellular fibrils (Am) in a hIAPP islet cultured at 28 mM glucose at two magnifications. Fibrils are oriented in parallel arrays in deep invaginations (arrows) of the beta-cell border as observed in man (18). G, secretory granule. IAPP released by exocytosis of IAPP-containing beta cell granules near the base of the invaginations of the cell membrane could polymerize onto the fibrils and thereby promote further extension of the membrane pocket into the beta cell. [Bar = 0.5 μm (*Left*) and 0.25 μm (*Right*).]

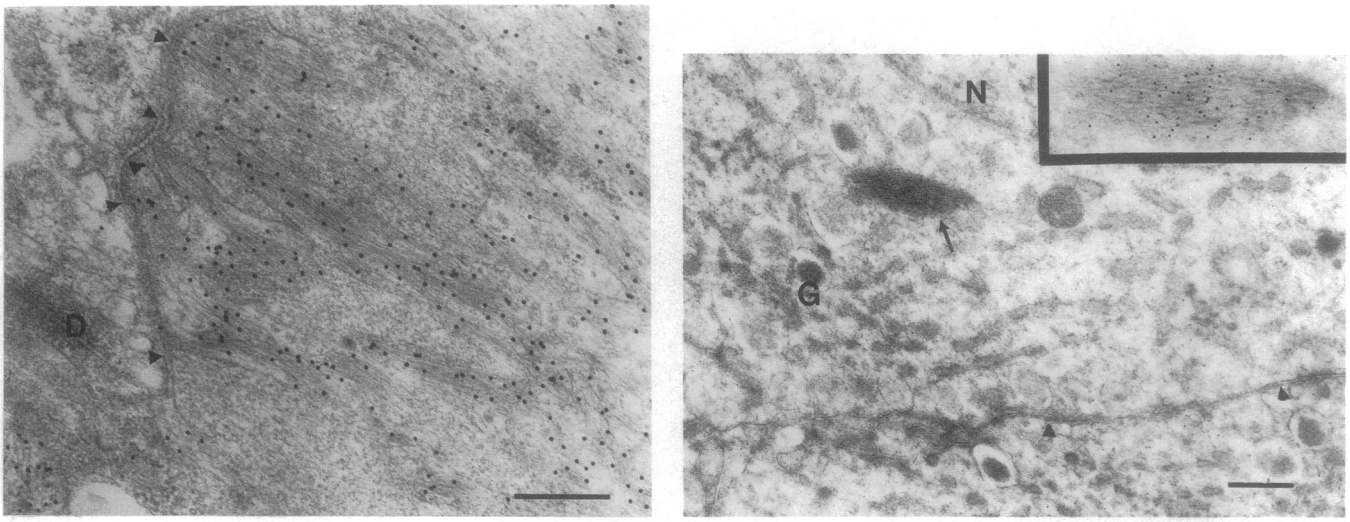

FIG. 4. (*Left*) Amyloid fibrils immunogold-labeled for IAPP occupy a large part of beta-cell cytoplasm in a hIAPP islet cultured at 28 mM glucose. Arrowheads indicate the cell border between two beta cells. Fibrils lie in bundles perpendicular to the cell membrane. D, desmosome. (Bar = 0.5 μm.) (*Right*) Islet beta cell of a hIAPP islet cultured at 11.1 mM glucose. Fibrils immunoreactive for IAPP (arrow) are present within the cytoplasm but are not associated with a particular organelle (*Inset*). G, secretory granule; N, nucleus; arrowheads, beta-cell border. No intracellular fibrils were observed in rIAPP or control islets. (Bar = 0.5 μm.)

beta cells does not appear to affect insulin release *in vitro*. It is therefore difficult to explain the mechanism by which synthetic exogenous IAPP has been shown to inhibit insulin secretion (15). The significance of intracellular fibril formation on beta-cell function is not known. The insulin response to glucose from hIAPP islets was similar to that from rIAPP and control islets (Fig. 1 *A* and *C*), suggesting that the presence of intra- or extracellular fibrils does not substantially influence beta-cell function over a short time period. However, the role of amyloid between beta cells and capillaries in the impairment of insulin release in NIDDM remains to be determined.

The absence of capillaries and basement membranes in isolated islets coupled with the presence of extracellular fibrils in hIAPP islets suggests that the basement membrane is not essential for amyloidogenesis as has been proposed (21). An amyloidogenic role for macrophages has also been postulated in many types of localized or generalized amyloidoses (22–24), but the lack of any association between macrophages and fibrils in cultured hIAPP islets indicates that polymerization of IAPP is not macrophage-dependent.

Our results show that hIAPP produced by beta cells *in vitro* will polymerize to form amyloid fibrils and can occupy large areas of beta-cell cytoplasm. Fibril formation is dependent upon activation of the transgene and secretion of hIAPP. Since secretion of IAPP in the pancreas appears to be coregulated with that of insulin (25), stimulation of pancreatic beta cells in NIDDM by obesity, hyperglycemia, and sulphonylurea therapy may promote amyloidogenesis and thus further aggravate the islet pathology. By manipulation of the tissue culture conditions for these transgenic mouse islets, it will be possible to determine the factors involved in amyloid formation that occurs over periods of weeks, rather than over decades as in patients with NIDDM.

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