Volume 323, number 1,2, 40–44 FEBS 12468 May 1993

Human islet amyloid polypeptide transgenic mice as a model of non-insulin-dependent diabetes mellitus (NIDDM)

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Received 22 March 1993

To model islet amyloidogenesis in NIDDM and explore the glucoregulatory role of islet amyloid polypeptide (IAPP), we have created transgenic mice containing a rat insulin-I promoter–human IAPP fusion gene. Expression of human IAPP was localized to the islets of Langerhans, anterior pituitary and brain in transgenic animals; blood IAPP levels were elevated 5-fold while fasting glucose levels remained normal. Amyloid deposits have not been detected in transgenic islets suggesting that other co-existing abnormalities in NIDDM may be required for the formation of islet amyloid. These animals provide a unique model for exploring this hypothesis and other proposed functions of IAPP.

Transgenic mouse; Islet amyloid polypeptide; Amylin; Non-insulin-dependent diabetes mellitus; Islet amyloid; Islet of Langerhans

1. INTRODUCTION

NIDDM or Type II diabetes mellitus, is characterized by peripheral insulin resistance, hyperglycemia, impaired insulin secretion and the development of amyloid deposits in the islets of Langerhans. The principle component of islet amyloid is a 37 amino acid peptide termed, islet amyloid polypeptide (IAPP), also known as diabetes-associated peptide or amylin, which shares 45% sequence identity with calcitonin gene-related peptide [1–4]. IAPP is synthesized as a larger precursor protein in the islet beta cells and is processed, stored and coreleased with insulin into the blood stream [5–8].

A major unresolved question concerns the causal relationship between islet amyloid formation and beta cell dysfunction in NIDDM [9–11]. Pancreatic amyloid is found in > 90% of NIDDM patients and to a lesser extent in the nondiabetic elderly. IAPP amyloid has also been found in nonhuman primates, cats and raccoons in association with diabetes but not in species such as rats, mice and dogs [9,10]. Recent in vitro studies have linked this species-specificity to interspecies differences in residues 20–29 of the IAPP sequence thus providing important clues to the structural requirements for amyloidogenesis [12,13]. Understanding the in vivo biological changes which result in IAPP amyloidogenesis, however, has proven more difficult.

A second and controversial area of interest in IAPP concerns the biologic function(s) of this peptide. Structurally IAPP has the features of an endocrine hormone and has been proposed to have glucoregulatory activities. IAPP has been reported to inhibit insulin secretion from the pancreatic islets in some studies [14–16] but not others [17–19]. IAPP has also been shown to inhibit insulin-stimulated glycogen synthesis in muscle strips in vitro, and to antagonize insulin action, induce impaired glucose tolerance and elevate plasma glucose levels in vivo [20]. However, these responses have primarily been observed using pharmacologic doses of IAPP; studies using physiologic amounts of IAPP in vivo have not confirmed these findings [21–23]. Other less well-characterized biologic activities of IAPP that have been reported include effects on calcium homeostasis [24], amnesia [25] and anorexia [26,27].

In light of the many unresolved questions concerning the biologic function(s) of IAPP and its potential role in NIDDM, we have created, and describe herein, a transgenic animal model in which the consequences of overexpressing human (amyloidogenic) IAPP in the islets of Langerhans and blood can be directly addressed.

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2. MATERIALS AND METHODS

2.1. *Transgenic mice*

The transgene used was created by fusing a 523 bp *Bam*HI-*Hin*dIII DNA restriction fragment encompassing the rat insulin-I gene promoter region upstream of a 7.7 kb genomic DNA fragment containing the complete human IAPP gene. A *Bss*HI restriction fragment containing the transgene insert (designated, RIIP-gIAPP) was gel-purified and microinjected into FVB/N mouse (Taconic, Germantown, NY) zygotes as previously described [28]. Eight founder animals were iden-

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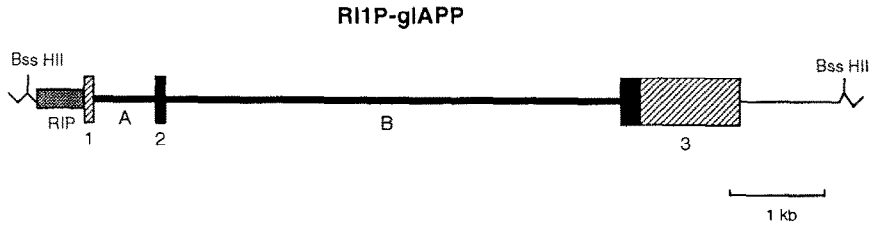

Fig. 1. RI1P-gIAPP transgene. The rat insulin-I gene promoter (RIP) and exons (1–3) and introns (A,B) of the huIAPP gene are indicated; 1.1 kb of 3′ flanking sequence is included in the construct (thin-lined region). 5′ and 3′ untranslated regions are hatched. *Bss*HII sites in the vector (jagged lines) were used for excision of the transgene insert for microinjection.

tified by Southern blotting, five of which yielded transgenic progeny. Three of these lines, #6, #13 and #16 tested positive for expression of the transgene and were used for the present studies.

2.2. *Northern blot analysis*

Total RNA was extracted from freshly isolated tissues according to the method of Chomczynski and Sacchi [29]. For Northern analysis, the RNA was electrophoresed on an 18% formaldehyde/1.5% agarose gel, blotted onto nitrocellulose and hybridized to a 329 bp IAPP cDNA probe labelled with ^{32}P by PCR [30]. The mouse insulin gene probe used consisted of a 60 bp, ^{32}P-labelled synthetic oligonucleotide.

2.3. *Western blot analysis*

Fresh tissues or isolated islets [31] were homogenized in 0.1 M NaCl, 1 mM EDTA, 0.01 M Tris-HCl (pH 7.6) plus protease inhibitors. After assaying for protein concentration (BioRad Lab., Cambridge, MA), the extracts were boiled, fractionated on a 4–20% Tris-glycine gradient gel (Novex, San Diego, CA) and electrophoretically transferred to 0.2 μm PVDF (Bio-Rad) or nitrocellulose (S&S) membrane. After incubating with primary antiserum for one hour, reactive protein bands were demonstrated using the enhanced chemiluminescence method (Amersham, Arlington Hts., IL). The primary antiserum was either rabbit anti-rat amylin/IAPP (Peninsula Labs, Belmont, CA) diluted 1:10,000 which detects both mouse (mu) and human (hu) IAPP (referred to here as anti-mu/huIAPP antiserum), or rabbit anti- human amylin/IAPP (Peninsula Labs) diluted 1:2,000 and pre-absorbed with synthetic rat (identical to mouse) IAPP (Peninsula Labs) to render the antiserum huIAPP-specific. Pre-absorbed antibody was prepared by incubating 0.4 ml of diluted (1:500) antiserum with 200 ng of peptide for 1.5 h at room temperature just prior to use. This latter antiserum is referred to here as anti-huIAPP.

2.4. *Histochemistry and indirect immunofluorescence (IIf)*

Six μm-thick frozen sections were fixed in PBS-buffered 2% paraformaldehyde for 30 min and processed for IIF as previously described [28]. The primary antiserum was either anti-mu/huIAPP (described above) or anti-huIAPP (described above). Secondary antibody was FITC goat anti-rabbit IgG (Cappel/Organon Teknika Corp., Durham, NC). Histochemical detection of amyloid was carried out on tissue sections using alkaline Congo red staining and subsequent examination under a polarizing microscope.

2.5. *Plasma IAPP, insulin and glucose analysis*

For plasma IAPP and insulin level determinations, 12–15 eight-week-old transgenic mice or non-transgenic (control) siblings, were fasted overnight and bled retro-orbitally into tubes containing anticoagulant (Sequester-Sol, Cambridge Chem. Prod. Inc., Fort Lauderdale, FL). Individual plasma fractions were isolated, pooled and aliquoted (1 ml for IAPP and 0.05 ml for insulin assay). The IAPP assay is described in detail elsewhere [32]. Briefly, the assay is based on rabbit anti-human IAPP antiserum (Peninsula, Meerseyside, UK) with labelled rat/mouse IAPP (Amersham) as tracer and detects changes between adjacent samples equivalent to 1 pmol/l plasma. Before assay, each plasma sample was extracted on a Sep-Pak C_{18} cartridge and the eluate was lyophilized and reconstituted in assay buffer. Insulin levels were determined using an RIA kit from Diagnostic Products Corporation (San Diego, CA) and rat insulin (Eli Lilly) as the standard. IAPP and insulin assays were run on 3–6 aliquots of plasma from each animal group. Blood glucose levels were determined on individual fasted mice (12–22 mice/group) by the glucose oxidase method using a model 300 Alpkem Rapid Flow Analyzer.

3. RESULTS

The hybrid transgene construct used for the generation of transgenic mice is shown in Fig. 1. Three stable lines of transgenic mice were created which expressed high levels of transgene mRNA in the pancreas by Northern analysis (Fig. 2). Transgene-derived mRNA transcripts observed included a major 1.6 kb transcript and several

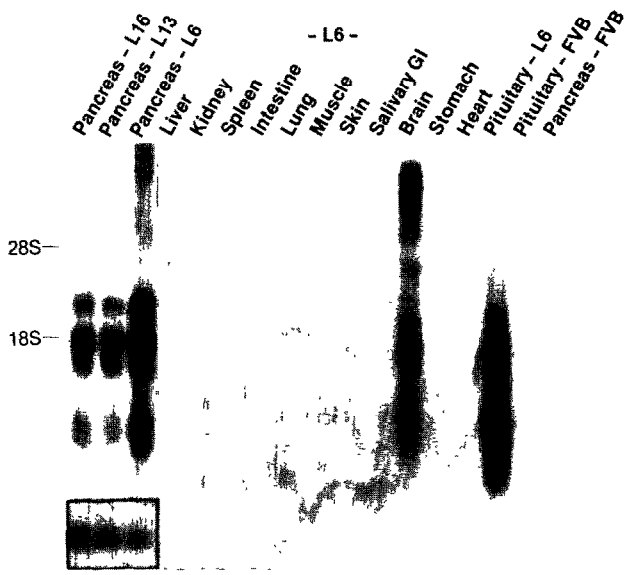

Fig. 2. Northern blot analysis. Forty μm of total RNA (15 μg in pituitary lanes) extracted from tissues of line (L) 6, 13 and 16 progeny and non-transgenic FVB mice was subjected to Northern blot analysis. Multiple transgene mRNA transcripts are detected in the pancreas in all three lines and in brain and pituitary of line 6. While not shown, line 13 and 16 mice also expressed the transgene in the brain and pituitary. Expression of endogenous mouse IAPP is below the level of detection in total pancreatic RNA using this probe. Inset: pancreatic RNA from line 13 and 6 transgenic mice (left 2 lanes) and a non-transgenic mouse (right lane) hybridized to a mouse insulin probe.

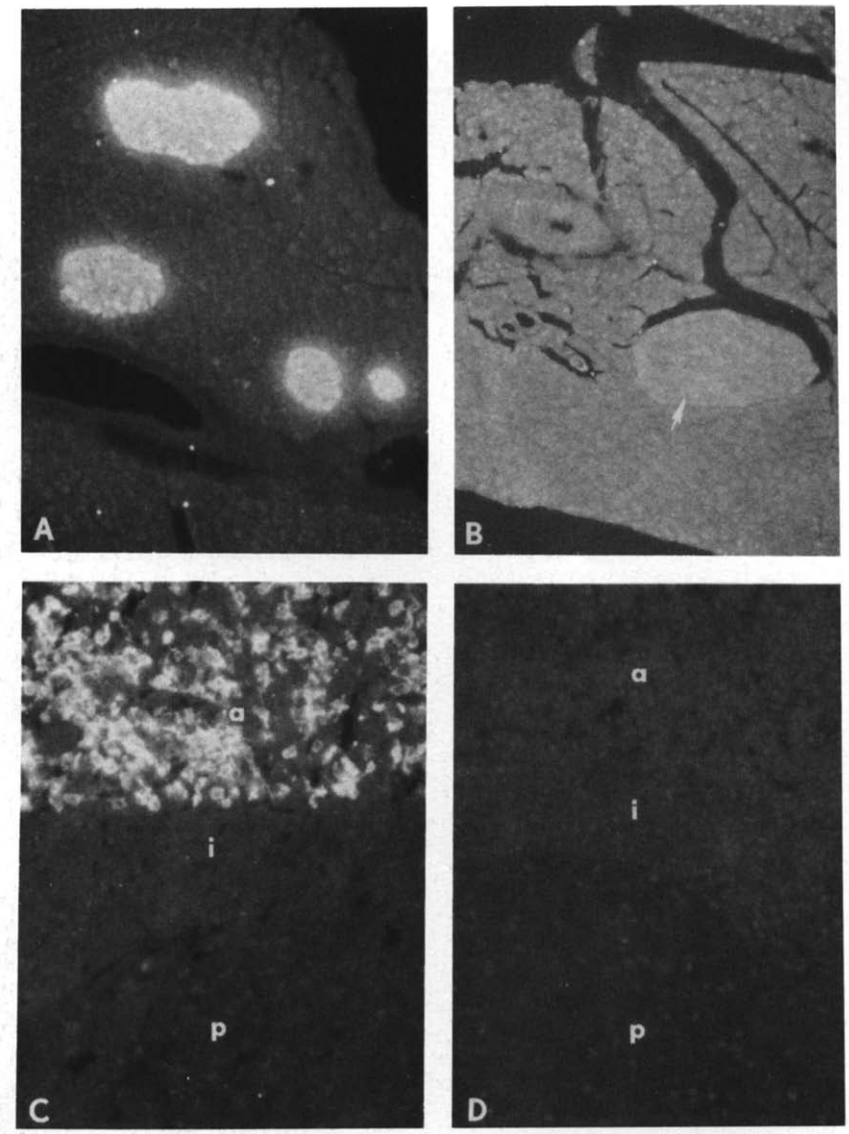

Fig. 3. Indirect immunofluorescence. (A) HuIAPP-specific antiserum reacts strongly with line 6 pancreatic islets but not with non-transgenic islets (B). Transgenic IAPP is also detected in a subpopulation of cells in the anterior ptuitary (a) of line 6 mice reacted with anti-mu/huIAPP but not the intermediate (i) or posterior (p) pituitary (C), nor is endogenous muIAPP detected in the pituitary of non-transgenic mice (D). All figures × 200.

less abundant transcripts which likely arise from multiple polyadenylation signals in the IAPP gene [33]. Transgene mRNA expression in the pancreas was highest in line 6 by this method of detection and judged to be roughly equivalent to the level of endogenous insulin gene expression (compare the major 1.6 kb transcript to insulin transcript in Fig. 2, inset) and at least ten-fold higher than that of endogenous mouse IAPP [34]. Significant extrapancreatic expression was also detected in the brain and pituitary gland in all three lines (illustrated for line 6 in Fig. 2); low levels of transgene expression were detected in the intestine and testes of some line 13 and 16 mice but not line 6 animals (not shown).

While expression of transgenic mRNA was detected in several tissues, expression of transgenic IAPP protein was only detected in the pancreatic islets of Langerhans and a subpopulation of cells in the anterior pituitary when localized by IIF (Fig. 3). The cellular localization of IAPP in these tissues appeared identical in all three lines. Since the pattern of reactivity in the transgenic islets was identical with either the huIAPP-specific antiserum and antiserum specific for rat/mouse IAPP (Fig 3; and N. Fox, unpublished findings), it is concluded that both endogenous mouse and exogenous human IAPP are being co-synthesized in the transgenic islet beta cells. The specific cell type(s) expressing the transgene in the anterior pituitary were not determined; immunoreactivity in the pituitary was not due to endogenous mouse IAPP (Fig. 3D). Finally, whereas intracellular immunoreactivity for IAPP was clearly pres-

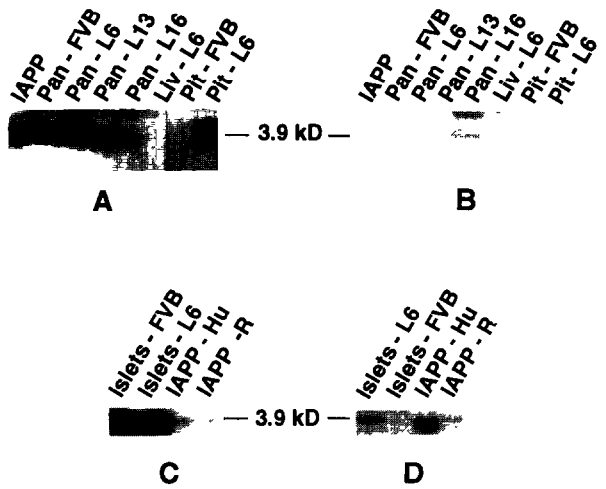

Fig. 4. Western blot analysis. (A) Thirty μm of protein/lane from pancreas (Pan), pituitary (Pit), and liver (Liv) of transgenic line (L) 6, 13 and 16 mice and non-transgenic control mice (C) were blotted and reacted with anti-mu/huIAPP antiserum to examine total IAPP levels. IAPP is readily detected as a 3.9 kDa protein species in lanes containing transgenic pancreas and pituitary, non-transgenic pancreas and synthetic IAPP (10 ng) but not transgenic liver or non-transgenic pituitary. (B) Same blot as in (A) reacted with anti-mu/huIAPP antiserum pre-absorbed with synthetic IAPP Reactivity with the 3.9 kDa IAPP band is completely abolished. (C) Blot of 10 mg of islet protein (= 10 islets) and 10 ng of human (Hu) and rat (R) IAPP standards reacted with anti-mu/huIAPP antiserum A small elevation in total IAPP is detected in transgenic islets. (D) Transgenic and non-transgenic islets reacted with the huIAPP-specific antiserum; the 3.9 kDa protein is only detected in the transgenic islet extracts.

ent in transgenic islets, no extracellular deposits of IAPP were detected either immunohistochemically or by histochemical staining of tissue sections with amyloid-specific Congo red (see section 2) in transgenic mice as old as one year of age.

A MW 3.9 kDa band corresponding to the processed form of IAPP was readily detected in protein extracts from transgenic or non-transgenic pancreata and transgenic pituitaries by Western blot analysis (Fig. 4). No significant difference in the levels of total pancreatic IAPP was apparent between transgenic lines or between transgenic and non-transgenics in total pancreatic extracts. To evaluate the IAPP levels more accurately, protein extracts from equal numbers of purified islets from line 6 and non-transgenic mice were compared. Here, total IAPP levels in transgenic islets appeared modestly elevated relative to non-transgenic islets (Fig. 4C). Consistent with the IIF results, the huIAPP-specific antiserum reacted with the MW 3.9 kDa protein in transgenic islet extracts but not in non-transgenic islet extracts (Fig. 4D), further establishing that fully processed huIAPP is being synthesized in the transgenic islets.

Table I

Plasma IAPP/insulin/glucose*

	IAPP (pmol/l)	Insulin (pmol/l)	Glucose (mg/dl)
Transgenic males	38.5 ± 2.4	200 ± 38	80 ± 4.2
Control † males	16.7 ± 2.5	233 ± 28	81.4 ± 4.2
Transgenic females	63.0 ± 2.4	183 ± 18	79.9 ± 2.3
Control females	11.3 ± 1.9	200 ± 27	77.6 ± 3.1

* Assays performed on age-matched fasted animals; data expressed as mean ± S.E.M.

† Controls: non-transgenic siblings.

Plasma IAPP, glucose and insulin levels were critically evaluated in fasted animals from line 6 mice which expressed the highest levels of transgene mRNA. These results are summarized in Table I. Whereas IAPP levels were elevated approximately 2-fold in transgenic males and 5-fold in transgenic females from this line, glucose and insulin levels remained unchanged when compared with sex- and age-matched non-transgenic siblings. Similar results have also been obtained in a smaller sampling of line 13 and 16 mice (N. Fox and G. Westermark, unpublished results). Why the plasma IAPP levels differ in transgenic male vs. female line 6 mice is uncertain and is under investigation. This may be due, for example, to a chromosomal position effect [35] whereby a particular transgene integration site may allow stronger transgene transcription in a given tissue in females relative to males.

4. DISCUSSION

The expression of the RI1P-gIAPP transgene in the pancreas and the brain in our transgenic animals is consistent with an earlier report that sequences from −346 to −103 bp flanking the insulin-I gene promoter will direct transgene expression to these sites [36]. Expression of the transgene in the anterior pituitary in all three lines was unexpected and may have resulted from the unmasking of cryptic regulatory elements in the human IAPP gene and/or a novel interaction between the rat insulin I gene promoter/flank and the IAPP gene. RI1P-gIAPP mRNA transcripts were also detected in the brain, yet no immunoreactive IAPP was detected here. Notably, the RI1P-gIAPP mRNA transcripts in brain were found to correspond to sense-strand (hence potentially translatable) huIAPP mRNAs by hybridization to sense and anti-sense riboprobes (G. Westermark, unpublished results). Thus, this suggests that translational or post-translational synthesis/processing of IAPP may be inefficient in this tissue.

There did not appear to be a major elevation of total IAPP protein in the islets despite a high level of transgene mRNA expression and IAPP in the blood. This could be due to a higher rate of IAPP release from the transgenic islets. Translational or post-translational regulatory mechanisms may also affect IAPP production as suggested in earlier experiments by our group [37].

The fold-increase in blood IAPP levels in female transgenic line 6 mice relative to control animals was significantly greater than the highest reported physiologic increase observed in man (i.e. 2–3-fold) following glucose challenge (reviewed in [11]). Despite this elevation, no change in blood glucose levels was evident as might have been expected if IAPP had a significant glucoregulatory function as proposed [20]. Failure to reveal such an effect was not due to a compensatory increase in insulin secretion as insulin levels were normal in the transgenics. Hence, under the present conditions, IAPP does not appear to exhibit significant glucoregulatory activity.

We have not detected the presence of amyloid deposits in the islets (or pituitary) by Congo red staining of histologic sections from mice as old as one year of age. Amyloid formation may require more time and/or a predisposing event such as concurrent diabetes. Since these conditions can be experimentally invoked and/or genetically introduced into mice by interbreeding with diabetes-prone *db/db* and *ob/ob* mice, these transgenics should provide a unique model for further exploration of the factors involved in islet amyloidogenesis and NIDDM.

Acknowledgements. We wish to thank Ray Carroll and the laboratories of Thomas Stephens, Gerald Gold, Terence Yen and William Heath for technical assistance and helpful discussions during the course of these studies. G.T.W. and P.W. were supported by the Swedish Medical Research Grant 5941.

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