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Rapid Communication

Impaired Glucose Tolerance is Associated with Increased Islet Amyloid Polypeptide (IAPP) Immunoreactivity in Pancreatic Beta Cells

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Adult cats determined by clinical laboratory evaluations to be normal, impaired glucose tolerant, or overtly diabetic were used to explore prospectively the relationships among pancreatic beta cell islet amyloid polypeptide (IAPP) immunoreactivity, islet amyloid (IA) deposition, and diabetogenesis. IAPP-derived IA was found in 11 of 14 (79%) diabetic cats, in four of nine (44%) impaired glucose tolerant cats, and in two of eight (25%) normal adult cats. The presence of IA even in very small amounts, therefore, predicts a very high probability (88%) that an animal has either impaired glucose tolerance or overt DM. Although all overtly diabetic cats had a marked decrease or absence of beta cell IAPP immunoreactivity, six of six cats with impaired glucose tolerance retained IAPP immunoreactivity with 1:15,000 dilutions of antisynthetic IAPP 7-17, whereas only one of seven normal cats had IAPP immunoreactivity beyond 1:10,000 dilutions. These findings suggest that increased IAPP production preceding the development of overt DM is linked to the progressive formation of insoluble IA deposits that are apparent in most overtly diabetic individuals. Of most importance, in that IAPP has been reported to inhibit both basal and insulin-stimulated rates of glycogen synthesis, is the possibility that increased production and release of IAPP by pancreatic beta cells plays a key role in the development of the insulin resistance and impaired glucose tolerance, both of which occur in Type 2 DM. (Am J Pathol 1989, 135:245–250)

The putative hormone identified as islet amyloid polypeptide (IAPP) was recently shown to be the predominant component of pancreatic islet amyloid (IA) deposits present in age-related diabetes mellitus (DM) of humans and cats.[1,2] This peptide has been immunohistochemically shown to be a normal component of pancreatic beta cells from many animal species,[3–5] and with immunogold labeling techniques has been found stored in beta cell granules for subsequent cosecretion with insulin.[5]

Although IAPP immunoreactivity is evident within beta cells of many animal species, deposition of IA in association with DM is known to occur in relatively few species (eg, humans, cats, nonhuman primates).[6–9] In all of these species, IA deposition is associated with age-associated (type 2 or noninsulin-dependent) DM but not juvenile (type 1 or insulin-dependent) DM. IA occurs in over 90% of human type 2 diabetics,[10] and in approximately 75% of adult diabetic cats.[9]

Although IA also occurs in humans[10] and cats[11] that do not have overt DM, the common concurrence of IA with age-related DM in humans and cats led us to examine the possibility that these deposits provide an important clue to the pathogenesis of this disease. The domestic cat, provides an excellent animal model[12] to explore the relationship of IA formation with diabetogenesis.

In the present investigation, cats known by clinical laboratory evaluations to be normal, impaired glucose tolerant, or overtly diabetic were used to explore the relationships among beta cell IAPP immunoreactivity, IA deposition, and diabetogenesis. The results provided important

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new evidence suggesting that increased production and release of IAPP by pancreatic beta cells in cats with impaired glucose tolerance is linked to the progressive deposition of IA and to development of impaired glucose tolerance that leads to overt DM. In contrast, overt DM was found to be associated with a marked reduction or absence of beta cell IAPP immunoreactivity and characteristically extensive IA deposits.

The results of this investigation are especially significant in light of recent evidence[13,14] indicating that IAPP (designated as amylin or diabetes-associated peptide [DAP]) is an inhibitor of both basal- and insulin-stimulated rates of glycogen synthesis in rat muscle. Increased expression of IAPP by pancreatic beta cells thus may play a key role in the development of the insulin resistance and impaired glucose tolerance that occur in type 2 DM.

Materials and Methods

Animals

Thirty-one domesticated cats (14 with DM, nine with impaired glucose tolerance, and eight normal controls) were used for various aspects of this study.

Spontaneously diabetic cats (N = 14) were obtained by owner donations through local veterinary practitioners. Overt DM was confirmed in each case by clinical history, urinalysis (persistant glucosuria), and fasting blood glucose levels (range, 270 to 625 mg/dl) using the glucose oxidase method. Diabetic cats ranged from 8 to 16 years of age.

Normal (N = 8) and impaired glucose tolerant cats (N = 9) were obtained by screening young adult cats (estimated to be between 2 to 5 years) obtained from the University of Minnesota Research Animal Resources Center. Each cat was acclimated to the animal holding facilities before evaluation with the high dose intraveneous glucose tolerance test using indwelling catheters and procedures previously described.[15] All glucose tolerance tests were done between 9 and 11 A.M. on conscious and unsedated cats. Linear regression analyses of a semilogarithmic plot of glucose concentration *versus* time was used to calculate the one half time for glucose disappearance (T½). Categorization of cats as impaired glucose tolerant or normal was based on our previously documented criteria.[15] Cats with T½ values more than two standard deviations above the normal mean were considered to have impaired glucose tolerance (ie, T½ values over 40 minutes).

Histochemistry and Immunohistochemistry

The pancreas from each cat was obtained immediately after euthanasia with an overdose of pentobarbital sodium. From each animal, 5-mm transverse pieces of pancreas were obtained from each major region of the pancreas (head, tail, and angle) and fixed in 10% neutral buffered formalin.

Table 1. *Occurrence of IAPP-Derived IA in Diabetic, Impaired Glucose Tolerant, and Normal Cats*

Cats	Cats with IA		Cumulative totals
	Present study	1985 study[15]	
Diabetic	11/14	6/7	17/21 (81%)
Impaired glucose tolerant	4/9	3/9	7/18 (39%)
Normal	2/8	0/7	2/15 (13%)

The occurrence of amyloid deposits in all 31 cats was evaluated using 4-μm-thick serial sections of paraffin-embedded pancreas stained with hematoxylin-eosin (H & E) and Congo red (CR). Three H & E-stained sections and 3 CR-stained sections from each major region of the pancreas (approximately 2000 total islets) were evaluated from each cat.

IAPP immunoreactivity was evaluated using the peroxidase antiperoxidase (PAP) method[16] in all 31 cats using 4-μm-thick serial sections of paraffin-embedded pancreas (head, tail, and angle) mounted on chromalum-coated glass slides. Rabbit antisynthetic undecapeptide corresponding to positions 7-17 of human IAPP (1:2000 dilution)[3] was used as the primary antiserum. Additionally, in seven normal cats and in six cats with impaired glucose tolerance, IAPP immunoreactivity was evaluated using the following dilutions of rabbit antisynthetic IAPP 7-17: 1:2000 (positive control), 1:10,000, 1:15,000, 1:20,000, and 1:25,000. In diabetic cats, consecutive sections of pancreatic islets were evaluated using antisynthetic IAPP 7-17 and antiporcine insulin (1:200 dilution) (Dako Corporation, Santa Barbara, CA). All PAP procedures using anti-IAPP 7-17 were performed under constant and uniform conditions, including overnight incubation (4 C) with primary antiserum, 20-minute incubation at room temperature with the linking antibody, 20-minute incubation with the PAP reagent, and 5-minute development times using the chromagen 3-amino-9-ethylcarbazole (AEC). Immunohistochemical negative controls included the use of primary antiserum absorbed with excess homologous antigen[3] and replacement of the primary antiserum with nonimmune rabbit serum.

Results

Islet Amyloid

The occurrence of islet amyloid (IA), substantiated by CR stains and polarization microscopy, was evaluated in approximately 2000 islets from each cat. IA was demonstrated (Table 1) in the islets of 11 of 14 (79%) diabetic

cats, four of nine (44%) cats with impaired glucose tolerance, and two of eight (25%) normal cats. IA in diabetic cats was generally present in more than 60% of the islets, and these deposits were moderate to heavy in extent. However, one diabetic cat had small IA deposits in less than 1% of its islets. IA deposits in cats with impaired glucose tolerance were, in contrast to those of diabetic cats, much less extensive with less than 2% of the islets being affected. Very small and inconspicuous IA deposits were present in only one or two islets from each of the two normal cats with IA.

IA deposits in all cats, regardless of clinical category, gave strong immunoreactivity with the PAP method when antisynthetic IAPP 7-17 was used as the primary antiserum.

Immunoreactivity of Islet Beta Cells with Anti-IAPP 7-17

Beta cells from all overtly diabetic cats nearly always lacked any evidence of IAPP immunoreactivity (Figure 1C, D). This observation was consistently apparent regardless of the extent of amyloid deposition. Beta cells with IAPP immunoreactivity were uncommon in all diabetic cats and, when observed, the intensity of immunoreactivity was very light compared with beta cells from normal cats.

Of 14 diabetic cats, 12 had beta cells with insulin immunoreactivity which, when compared with those of normal cats, was usually reduced with respect to intensity and number of immunoreactive cells. A total lack of insulin immunoreactivity was apparent in islets of only two of the diabetic cats. Islet cells of these two cats also lacked evidence of IAPP immunoreactivity.

Cats with impaired glucose tolerance invariably had beta cells with strong IAPP immunoreactivity (Figure 1E, F). The intensity of beta cell IAPP immunoreactivity in these cats commonly exceeded the intensity of immunoreactivity manifested by adjacent amyloid deposits in the same islets (Figure 1E). Comparison of the intensities of IAPP immunoreactivity between beta cells from normal cats and cats with impaired glucose tolerance provided some subjective, but not quantifiable, evidence for more intense IAPP immunoreactivity in beta cells of the impaired cats. Using more quantifiable methods, this premise was further tested in seven normal cats and six cats with impaired glucose tolerance. In these animals (selected for having identical 6-hour pancreas fixation times), the PAP method was repeated using a series of incrementally increasing dilutions (1:2000, 1:10,000, 1:15,000, 1:20,000, and 1:25,000) of antisynthetic IAPP 7-17 under constant and uniform conditions. As Table 2 shows, beta cells from all the cats tested elicited IAPP immunoreactivity through the 1:10,000 dilution. At the 1:15,000 dilution, however, six of six cats with impaired glucose tolerance and only one of seven normal cats retained IAPP immunoreactivity. Beta cells of all cats lacked evidence of IAPP immunoreactivity at dilutions of 1:20,000 or 1:25,000.

Discussion

The recent discovery of a previously unknown pancreatic islet polypeptide, identified as IAPP[1,2] (or DAP, diabetes associated peptide[17]), has provided new directions for investigations concerning DM association with aging. This polypeptide, which was shown to be the major polypeptide constituent of IA isolated from type 2 diabetic humans and spontaneously diabetic cats, is 37 amino acids in length and has over 40% sequence identity with the neuropeptide calcitonin gene-related peptide (CGRP).[18,19]

The very common occurrence of IAPP-derived IA deposits in type 2 human diabetes and spontaneously diabetic cats raises the possibility that aberrations in IAPP metabolism or catabolism are linked to both IA formation and diabetogenesis. Earlier[20] we proposed that the presence of IAPP-derived IA deposits signals a significant and early transition from normal to abnormal islet function, ie, the polymerization of IAPP to form amyloid fibrils provides a morphologic signal of early islet cell dysfunction. The demonstration of diminished IAPP immunoreactivity in beta cells of humans with type 2 DM supports this.[3]

The cat provides a unique animal model to study prospectively the sequential nature of IAPP production and its relationship to the development of IA and impaired glucose tolerance or overt DM. In this investigation, we used cats that were established by laboratory analyses to be normal, impaired glucose tolerant, or overtly diabetic. The occurrence of IA deposition and degree of IAPP expression by beta cells was carefully evaluated immunohistochemically in all cats from each of these three groups.

The cumulative results of this and our previously reported study[15] show that IAPP-derived IA deposition is strongly associated with the progressive development of impaired glucose tolerance and overt DM (Table 1). We found that 17 of 21 (81%) diabetic cats, seven of 18 (40%) impaired glucose tolerant cats, and two of 15 (13%) normal cats had IA deposits. Thus, the presence of IA (even in very small amounts) means there is a very high probability (92%) that an animal has either impaired glucose tolerance or overt DM.

The results of this immunohistochemical study, using a series of increasing antisynthetic IAPP 7-17 dilutions, suggest a possible link between the production and/or secretion of IAPP by beta cells and IA formation. Beta cells from six of six cats with impaired glucose tolerance re-

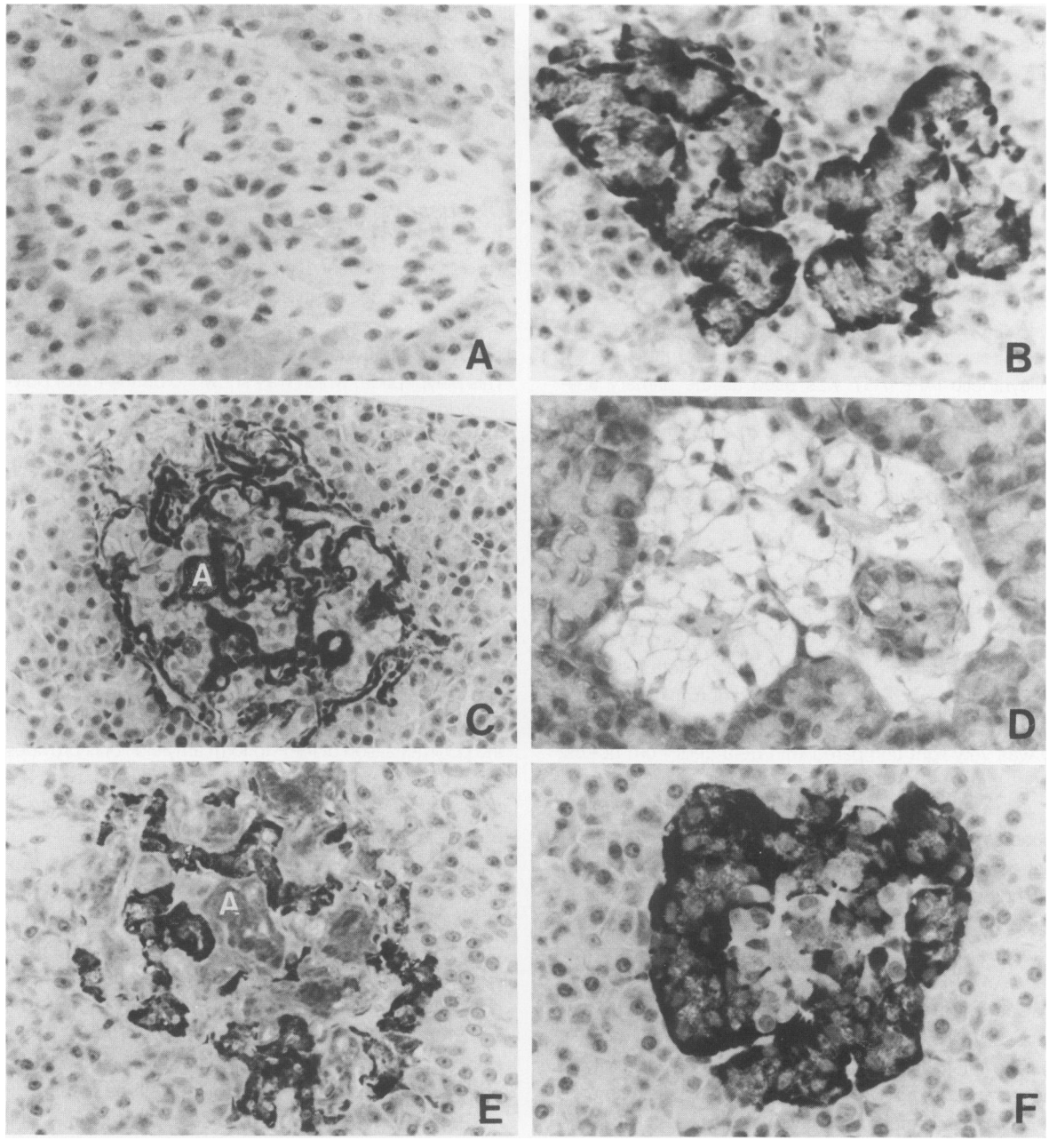

Figure 1. *Immunoreactivity of cat pancreatic islets using the PAP method and antisynthetic IAPP 7-17.* **A:** *Negative control, with the primary antiserum replaced with nonimmune rabbit serum.* **B:** *IAPP immunoreactivity of an islet from a normal cat.* **C:** *Amyloid-containing islet from a diabetic cat. Intense IAPP immunoreactivity is associated with bands of intercellular amyloid deposits (A) but islet cells lack any evidence of immunoreactivity.* **D:** *Diabetic cat islet that does not contain amyloid. The islet cells lack any evidence of IAPP immunoreactivity.* **E, F:** *Islets from cats with impaired glucose tolerance. Intense IAPP immunoreactivity is associated with islet beta cells. The intensity of beta cell immunoreactivity exceeds that of the adjacent intercellular amyloid (A) deposits (E) (×225). Hematoxylin counterstain.*

tained IAPP immunoreactivity at a 1:15,000 dilution of primary antiserum, whereas only one of seven normal cats had IAPP immunoreactivity at this dilution. Thus, an apparent increase in IAPP content of pancreatic beta cells was associated with impaired glucose tolerance and an increased incidence of IA deposition. The increase in IAPP content in cats with impaired glucose tolerance may be due to increased production of IAPP by the beta cells. An increase in production of IAPP would result in either a greater concentration of this peptide within the beta cell secretory vesicles, or increased numbers of vesicles, as IAPP is copackaged with insulin in the beta cell secretory vesicle.[5] Alternatively, the increase in beta cell IAPP content may be due to decreased secretion. Impaired first

Table 2. *Comparison of IAPP Immunoreactivity (PAP) in Normal and Impaired Glucose Tolerant Cats Using Increasing Dilutions of Anti-IAPP 7-17*

	Anti-IAPP 7-17 dilutions				
Cats	1:2,000*	1:10,000	1:15,000	1:20,000	1:25,000
Normal					
FIA 202	+	+	−	−	−
FIA 205	+	+	−	−	−
FIA 206	+	+	−	−	−
FIA 208	+	+	−	−	−
FIA 212	+	+	−	−	−
FIA 216	+	+	−	−	−
FIA 218	+	+	+	−	−
Impaired glucose tolerant					
FIA 203	+	+	+	−	−
FIA 213	+	+	+	−	−
FIA 225	+	+	+	−	−
FIA 230	+	+	+	−	−
FIA 235	+	+	+	−	−
FIA 236	+	+	+	−	−

* Positive control.
FIA = feline islet test.

phase insulin secretion in response to intravenous glucose has been shown in cats with impaired glucose tolerance.[15] However, most of these cats also exhibit increased second phase secretion, which tends to compensate for the lower first phase insulin secretion. Further study is needed to determine the mechanism for increased beta cell IAPP content and the association of this phenomenon with impaired glucose tolerance and IA deposition.

In this study, we also noted a marked decrease or absence of IAPP immunoreactivity in beta cells of all overtly diabetic animals. This dramatic decrease in IAPP immunoreactivity by beta cells in diabetic cats is consistent with our previous observations[3] that beta cells of type 2 human diabetics also have markedly reduced immunoreactivity with antiserum to synthetic IAPP. The marked decrease or absence of IAPP immunoreactivity observed in islets of diabetic cats is not due simply to an absence of beta cells. We have shown in previous studies,[21] for example, that the mean beta cell volume fraction in diabetic cats is reduced by approximately half. Also, in the present study, islets from 12 of 14 diabetic cats retained insulin immunoreactivity. Although the intensity of insulin immunoreactivity in individual beta cells generally decreased compared with that of normal cats, IAPP immunoreactivity in these same cells was nearly always absent.

A reduction in beta cell IAPP immunoreactivity in diabetic cats seems paradoxical in view of the tendency of diabetic cats and type 2 human diabetics to have large amounts of IAPP-derived and amyloid deposited in their islets. However, increased IAPP production, secretion, or both may precede the onset of overt diabetes mellitus, as the data from the impaired glucose tolerant cats seems to support; most of the amyloid deposition may, in fact, occur before overt diabetes. A more definitive explanation for our immunohistochemical findings in both humans[3] and cats must await data regarding IAPP expression and release, and also data regarding plasma levels of IAPP in normal conditions and in type 2 DM.

Recent molecular biological studies from our laboratory[22] and another laboratory[23] indicate that the entire 37 amino acid sequence of IAPP isolated from IA is identical with the predicted IAPP sequence obtained from cDNA sequencing. The results of these cDNA studies indicate that IAPP-derived IA deposits are not likely to be related to secretion of variant molecular forms of IAPP or to aberrant enzymatic processing of IAPP. On the basis of the present study, however, it is interesting to speculate that the polymerization of IAPP to form IA is more likely linked to increased synthesis and a subsequent increase in the localized concentration of IAPP.

Of most importance is the finding that increased production of IAPP by beta cells may play a significant role in the genesis of the impaired glucose tolerance observed before and during type 2 DM. Recent *in vitro* studies[13,14] indicate that IAPP is an inhibitor of both basal, and insulin-stimulated rates of glycogen synthesis in rat muscle. Increased production and release of IAPP by pancreatic beta cells may, therefore, play a key role in the development of the insulin resistance (ie, reduced blood glucose clearance and impaired glucose tolerance) that is seen in type 2 DM.

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