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Pro Islet Amyloid Polypeptide (ProIAPP) Immunoreactivity in the Islets of Langerhans

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ABSTRACT

Islet amyloid is typically found in type 2 diabetes mellitus and is believed to participate in the beta cell deterioration. The islet amyloid fibril consists of the 37-amino-acid islet amyloid polypeptide (IAPP) but its pathogenesis is only partly understood. We developed several different rabbit antisera against the flanking peptides of the IAPP precursor (proIAPP) and the proIAPP processing sites in order to study the possible occurrence of unprocessed proIAPP or parts thereof in islet amyloid. We applied these antisera in an immunohistochemical study on islet amyloid deposits present in a newly generated mouse strain that over-expresses human IAPP but is devoid of mouse IAPP. Male mice of this strain develop severe islet amyloidosis when given a high fat diet. Generally, the antisera showed no immunoreactivity with the amyloid. However, in scattered single beta cells, where amyloid could be seen intracellularly, immunoreactivity with one or more of the antisera colocalized with the amyloid. Although virtually all amyloid in human islets of Langerhans is found extracellularly, we propose that the initial amyloid formation occurs intracellularly, perhaps by not fully processed or folded (pro)IAPP. This amyloid, which may develop rapidly under certain circumstances, probably leads to cell death. If not degraded these amyloid spots may then act as nidus for further amyloid formation from fully processed IAPP, secreted from surrounding beta cells.

INTRODUCTION

Islet amyloid polypeptide (IAPP; also called "amylin") is a beta cell product occurring in mammals and birds. It is stored together with insulin in the secretory granules and the two substances are released together at exocytosis. In the human islets and plasma, the IAPP concentration is only a few percent of that of insulin on a molar basis.

IAPP is a 37 amino acid residue C-terminally amidated polypeptide. It belongs to

the calcitonin family and is almost 50% identical to the neuropeptide calcitonin gene related peptide (CGRP), from which it differs mainly in a central segment. In some species (primates, cats and raccoon), IAPP is known to give rise to amyloid deposits in the islets. The fibril formation capacity seems to reside in a small central motif of the IAPP molecule and, specifically, the sequence GAILS (residues 25–29) is important [28].

The function of IAPP is still a matter of discussion [11]. Many different effects on several organ systems have been described. Thus IAPP may be an important regulator of food intake and on gastric emptying. Among the most studied effects of IAPP are those on the glucose metabolism. Thus, IAPP exerts an insulin antagonistic effect on the skeletal muscle and induces insulin resistance in vivo when given in very high doses. IAPP's role as an inhibitory autocrine or paracrine regulator on islet beta and alpha cells is, however, better established.

IAPP was discovered through its common occurrence as amyloid depositions in the islets of Langerhans and in pancreatic beta cell tumors [3, 31, 32, 33]. Amyloid formation depends on a specific aggregation of usually small proteins or polypeptides where a transition to beta sheet conformation is believed to take place and a cross-fibril intermolecular beta sheet bonding is characteristic of all kinds of amyloid. About 20 different proteins are known to cause amyloid formation in human and among these there are so far five polypeptide hormones. The cause of amyloid fibril formation is poorly understood for all these proteins but several mechanisms may be important. Cleavage of a larger precursor may expose a normally hidden hydrophobic and amyloidogenic part of the molecule. Point mutation may create a more amyloidogenic protein or make it more susceptible to cleavage. Other components, e.g. heparan sulfate proteoglycan or apolipoprotein E, known to occur in amyloid deposits, may act as "pathological chaperones" and facilitate fibril formation. Modification such as glycosylation or glycation may also be important in making some amyloid proteins amyloidogenic. Finally, normal protective mechanisms against aggregation may be lost and cause amyloid fibril formation. Any of these mechanisms may be important in islet amyloid formation although mutation in the IAPP gene only is a rare event [15, 20].

IAPP is expressed as a prepromolecule [16]. ProIAPP is a 67 amino acid long propeptide, which undergoes enzymatic processing during its way to maturation into the biological active 37 amino acid polypeptide. Insulin is processed by the endoproteases PC1/3 and PC2 [12] and the co-localization of IAPP with insulin in the beta-cell granules has suggested that the enzymes responsible for the processing of insulin might also cleave IAPP. This process takes place in the secretory pathway during a pH decrease. Hitherto processing of human proIAPP by PC2 has been shown to occur in an in vitro translation system [1]. In the secretory granules insulin is localized to the core region while IAPP is localized together with the C-peptide to the halo-region [30]. In in vitro studies it was shown that insulin can abolish fibril formation of IAPP in a concentration dependent fashion [8, 30]. It is also possible that defective processing or folding can result in a more amyloidogenic IAPP-species that gives rise to amyloid. It has been shown in another form of polypeptide

hormone-derived amyloid, calcitonin-amyloid, that prohormone may constitute at least part of the amyloid [21].

The results concerning the possible occurrence of proIAPP or its flanking peptides in amyloid are somewhat contradictory. Only full-length IAPP has been identified by amino acid sequence analysis in human and cat amyloid deposits. However, we have earlier shown in an immunohistochemical study that the N-terminal fragment of IAPP can occur in islet amyloid [29]. Others have failed to find the C-terminal flanking peptide in islet amyloid [2]. Interestingly, however, it has also been shown that proIAPP can form amyloid like fibrils in vitro [7].

In this study, we have developed rabbit antisera against the flanking peptides of proIAPP and the proIAPP processing sites. In order to study early amyloid development, we have used these antisera in an immunohistochemical investigation of islet amyloid in a transgenic mouse strain that lacks endogenous IAPP but expresses human IAPP.

MATERIAL AND METHODS

Tissues. Formaldehyde (4%) fixed, paraffin-embedded normal human pancreatic tissue was available from the laboratory files. A mouse strain, expressing human IAPP (hIAPP) but not mouse IAPP (mIAPP) has been described [24]. Shortly, this strain was obtained by crossing a hIAPP transgenic mouse strain [16] with a IAPP null mutant (mIAPP-/-) mouse strain [5]. When given a high-fat diet, male hIAPP+/+mIAPP-/- mice develop severe islet amyloidosis beginning at the age of 9 months. Pancreatic tissue from such mice were fixed in 4% formaldehyde and embedded in paraffin.

For comparison normal human pancreatic tissue, embedded in Unicryl (Biocell, Cardiff, UK), was also available.

Synthetic peptides. Full-length human IAPP was synthesized at Keck Facility, Yale University, New Haven, CT. C-terminally amidated peptides corresponding to different parts of the proIAPP molecule were synthesized by t-Boc or f-moc chemistry (Figure 1). These peptides were all analyzed by reversed phase high performance liquid chromatography and mass spectrometry and showed expected masses.

Antisera. Peptides were coupled to keyhole limpet hemocyanin as described [6] and antisera were raised in rabbits by standard techniques. The production of antibodies was analyzed by enzyme-linked immunosorbent analysis (ELISA) and by immunohistochemistry [6].

Specificity studies. The specificity of the antisera was studied by ELISA and by slot blot analysis. For ELISA, the different peptides were dissolved in 50 mmol/l sodium carbonate buffer, pH 9.0 at a concentration of 50 μg/ml and tested against the antisera, diluted 1:100 and 1:200 as described [18]. For slot blot analysis, peptides were dissolved in carbonate buffer and 0.1 μg or 1 μg peptide were applied to a nitrocellulose membrane with the aid of a slot blot devise (Millipore). The membrane was blocked in 5% dry milk in 0.05 M Tris buffer containing 0.15 M sodium chloride (TBS) and incubated overnight at room temperature with the primary anti-

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-33                                1                                     37            +19
MGILKLQVFLIVLSVALNHLKATPIESHQVEKRKCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTYGKRNAVEVLKREPLNYLPL
Human IAPP                         KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY                   A110
Pep 74            NHLKATPIESHQV                                                            A102
Pep 514                    HQVEKRKCNT                                                      A164
Pep 582                    HQVEKRKC                                                        A169
Pep 515                                                            NTYGKRNAVE              A165
Pep 276                                                                 NAVEVLKREPLNYLPL A142
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Figure 1. Amino acid sequences of the different synthetic peptides used in this study. The peptide designation is to the left and the corresponding antiserum to the right. The upper sequence is that of preproIAPP.

sera diluted 1:500 in TBS. The membrane was washed, incubated with swine anti-rabbit horseradish peroxidase. The reaction was visualized with enhanced chemiluminiscense (Amersham-Pharmacia, Uppsala, Sweden).

Immunohistochemistry. Deparaffinized sections were incubated with the primary antisera diluted 1:250 over night at room temperature. For antigenic retrieval, treatment with hot 0.01 M sodium citrate buffer, pH 6.0, was used [14]. Primary antibody binding was detected with biotinylated swine anti rabbit antibodies followed by incubation with peroxidase-labeled streptavidine and the reaction was visualized with 3,3'diaminobenzidine tetrahydrochloride (DAB) as described [22]. Sections were counterstained with hematoxylin. Some sections were counterstained with alkaline Congo red B-solution [19] and studied in polarized light for simultaneous demonstration of amyloid and immunoreactivity. The specificity of the reactions was verified by replacement of the primary antisera with normal rabbit antiserum and by absorption of the primary antisera with corresponding antigen.

Immune electron microscopy. Immune electron microscopy was performed as described previously [25].

RESULTS

Specificity of antisera. All rabbits produced antisera strongly reacting with the uncoupled immunogen in ELISA. All these antisera were tested against the different antigens in order to evaluate possible crossreactions and the results are shown in Table 1. Antisera 102 and 165 exhibited no crossreactivity with IAPP while antiserum 164 showed a significant crossreactivity with IAPP, obviously due to overlapping sequences between IAPP and pep 514. The slightly elevated ELISA values of antiserum 142 with IAPP and pep 515 are probably insignificant since the slot blot analysis did not indicate cross-reactivity.

Reaction of antisera with normal human pancreas. The reactivity of the antisera with normal human pancreas was studied by immunohistochemistry and immune electron microscopy. Immunohistochemically, antisera 102 and 142 (Figure 2), both labeled beta cells in a pattern resembling labeling with antiserum to IAPP. Antisera 165 and 169 against peptides corresponding to the processing sites of proIAPP re-

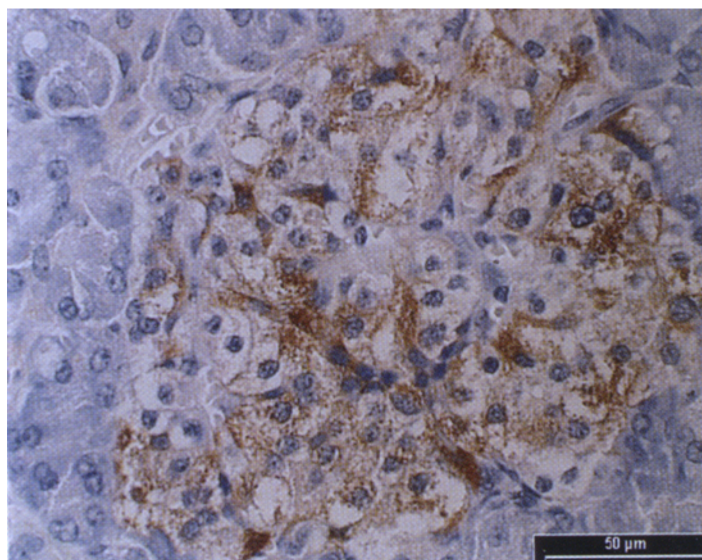

Figure 2. Normal human pancreatic islet immunolabeled with antiserum A142 against peptide 276. A beta cell labeling of varying strength is evident. Counterstained with hematoxylin.

vealed an usually perinuclear immunoreaction. This may indicate a trans Golgi accumulation similar to the situation with proinsulin [4]. Antiserum 164 labeled beta cells in a pattern similar to IAPP antibodies. Citric acid pretreatment increased the immunoreactivity with antiserum 169 only. The pre immune sera showed no labeling and absorption of the antisera with the corresponding uncoupled antigen abolished the reactions.

When used in immune electron microscopy, antisera 102, 142 and 165 showed a specific labeling exclusively of beta cell granules. Labeling of the individual granules

Table 1. Reaction in ELISA of synthetic peptides corresponding to different parts of human proIAPP with rabbit antisera raised against these peptides

	Antisera				
Antigen	102	169	164	165	142
IAPP	n.d.	0.09	0.63	0.06	0.15
Pep 74	2.30	0.02	0.02	0.02	0.07
Pep 582	0.08	0.49	0.16	0.04	0.06
Pep 514	0.09	0.09	1.91	0.08	0.00
Pep 515	0.09	0.06	0.00	1.09	0.17
Pep 276	0.00	0.04	0.01	0.03	1.60

n.d. = not determined

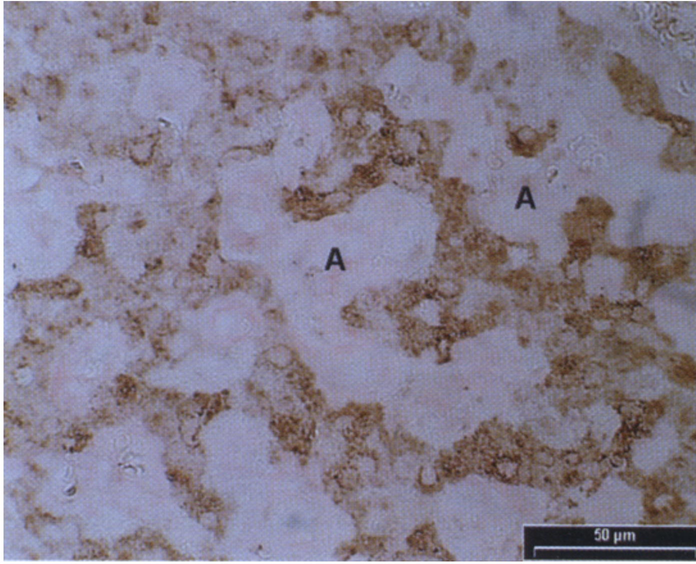

Figure 3. Part of a pancreatic islet of a hIAPP++mIAPP—mouse, immunolabeled as in *Figure 2.* The beta cells are labeled while the extracellular amyloid (A) is completely free of labeling. Counterstained with Congo red.

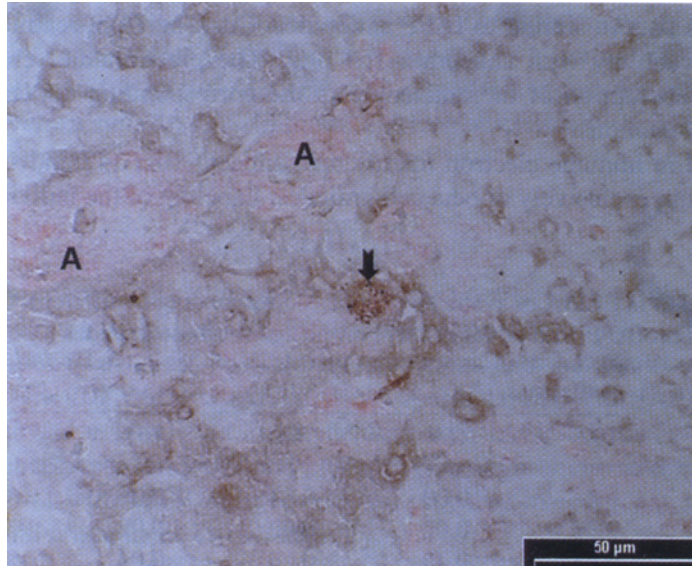

Figure 4. Part of a pancreatic islet of a hIAPP++mIAPP—mouse, immunolabeled with antiserum A102 against peptide 74. Amyloid (A) is unlabeled while one single cell (arrow), contains obviously intracellular amyloid which reacts with the antiserum. Counterstained with Congo red.

varied considerably with antiserum 142. Antiserum 169 labeled beta cell granules only weakly. Antiserum 164 was not tested due to the cross-reactivity with IAPP.

Reaction of antisera with pancreatic islets of hAIPP+/+mIAPP-/- mice. Pancreatic islets were studied in 9–12 month old mice with severe islet amyloidosis. The amyloid, which showed affinity for Congo red and green birefringence, occurred as massive deposits perivascularly and separated thereby the epithelial cells from the small blood vessels in the same fashion as seen in human type 2 diabetes. In few islets, single cells occurred in which the cytoplasm appeared to be filled with amyloid. Such cells had a pyknotic nucleus.

Antiserum to IAPP labeled beta cells and islet amyloid. Antisera 102 and 142 labeled beta cells similar to what was seen with anti IAPP. Antisera 165 and 169 labeled more scattered cells in an irregular pattern. With both these antibodies, a clear tendency to perinuclear labeling was evident.

The amyloid was generally not labeled with any of the antibodies 102, 142, 165 or 169 (Figure 3). However, some intracellular amyloid was labeled with antisera 142, 165 and 169 (Figure 4). Extracellular amyloid was labeled in only small and scattered areas with antiserum 102 and 142 but not with any of the other antisera against short peptides. Most of the extracellular amyloid was completely devoid of immunoreactivity with antisera to the flanking peptides or the processing sites (Figure 3).

DISCUSSION

We wanted to develop antisera to study the presence of proIAPP or its flanking peptides in islet amyloid. Such antisera would also be useful for the study of intracellular trafficking of IAPP. With the aid of short synthetic peptides, we were able to raise specific rabbit antisera to the flanking peptides and to the processing sites of the human proIAPP molecule. It was evident from the results with the two short peptides corresponding to the N-terminal processing site of proIAPP that even very short segments of overlapping can cause significant crossreactivity with mature IAPP. Thus, pep 514 has the KCNT sequence of the N-terminus of IAPP and antiserum to this peptide crossreacted with IAPP. Antiserum 169 against pep 582, which is two residues shorter than pep 514 and has only the KC-sequence of IAPP, showed no crossreactivity with either IAPP or its N-terminal flanking peptide. Surprisingly, antiserum 169 did not crossreact with pep 514.

All these antisera labeled normal human beta cells immunohistochemically and at the electron microscopical level, the labeling was confined to the secretory granules in which the peptides should occur at the highest concentration. The labeling was weakest with the antisera to the processing sites, most likely due to the low concentration of proIAPP in beta cells.

To investigate the possible presence of proIAPP or its flanking peptides in islet amyloid deposits we chose to use a transgenic mouse strain in which males given high fat diet develop severe islet amyloid as early as at 9 months of age. We reasoned that this amyloid should better reflect early events in amyloidogenesis

compared to the human islet amyloid deposits that we study in post mortem material. Evidence for this was the finding of intracellular amyloid in the mouse islets, something that is also seen when islet amyloid develops rapidly in human islets transplanted into nude mice [27] and in human insulinomas [17] but not in human islets of type 2 diabetic individuals [26]. Interestingly, we found that islet amyloid was usually devoid of immunolabeling with any of the four antisera against the flanking peptides of proIAPP or the proIAPP processing sites, with one exception. Thus, few single cells with intracellular amyloid showed colocalization of congophilic material and immunolabeling for propeptides and processing sites. This finding may allow us to propose the following initiating mechanism for the development of islet amyloid.

Formation of amyloid fibrils can be imitated in vitro by the use of synthetic amyloid fibril proteins, including IAPP. From such studies it has become evident that fibril formation occurs only after a significant lag phase during which a nidus is believed to build up [10]. However, if the peptide solution is seeded with a small proportion of pre-formed fibrils, there is almost no lag phase. A common finding in the human pancreas is the occurrence of islets that are almost completely converted into amyloid while other may be completely free of amyloid. Thus, it is tempting to speculate that a nidus formation for amyloid protein aggregation has occurred in such islets with amyloid only. We suggest that this nidus may develop by intracellular aggregation of proIAPP or its derivatives. This intracellular proamyloid leads to cell death, probably by apoptosis [9, 13] which ultimately will leave a small extracellular fibrillar residue on which extracellular amyloid develops. If this hypothesis is correct, the most important event in amyloidogenesis may well occur within the beta cell and not extracellularly, probably during a limited period of time. Obviously, the intracellular processing and trafficking of (pro)IAPP in the beta cell deserves more study.

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