Matrix Metalloproteinase-9 Reduces Islet Amyloid Formation by Degrading Islet Amyloid Polypeptide*

Kathryn Aston-Mourney†§1, Sakeneh Zraika†§, Jayalakshmi Udayasankar†§2, Shoba L. Subramanian†§, Pattie S. Green†§, Steven E. Kahn†§, and Rebecca L. Hull†§§

From the †Veterans Affairs Puget Sound Health Care System, Seattle, Washington 98108, the ‡Department of Medicine, University of Washington, Seattle, Washington 98195, and the §Metabolic Research Unit, School of Medicine, Deakin University, Waurn Ponds, 3216 Victoria, Australia

Background: Aggregation of IAPP is toxic to pancreatic islet β-cells.

Results: MMP-9 cleaves IAPP, preventing its aggregation and toxicity; islet MMP-9 mRNA levels are reduced in type 2 diabetes.

Conclusion: Reduced islet MMP-9 may contribute to amyloid-induced β-cell loss in type 2 diabetes.

Significance: MMP-9 is a novel mediator of IAPP metabolism and a potential target to limit amyloid formation in diabetes.

Deposition of islet amyloid polypeptide (IAPP) as amyloid is a pathological hallmark of the islet in type 2 diabetes, which is toxic to β-cells. We previously showed that the enzyme neprilysin reduces islet amyloid deposition and thereby reduces β-cell apoptosis, by inhibiting fibril formation. Two other enzymes, matrix metalloproteinase (MMP)-2 and MMP-9, are extracellular gelatinases capable of degrading another amyloidogenic peptide, Aβ, the constituent of amyloid deposits in Alzheimer disease. We therefore investigated whether MMP-2 and MMP-9 play a role in reducing islet amyloid deposition. MMP-2 and MMP-9 mRNA were present in mouse islets but only MMP-9 activity was detectable. In an in vitro culture model where human IAPP (hIAPP) transgenic mouse islets develop amyloid but nontransgenic islets do not, a broad spectrum MMP inhibitor (GM6001) and an MMP-2/9 inhibitor increased amyloid formation and the resultant β-cell apoptosis. In contrast, a specific MMP-2 inhibitor had no effect on either amyloid deposition or β-cell apoptosis. Mass spectrometry demonstrated that MMP-9 degraded amyloidogenic hIAPP but not nonamyloidogenic mouse IAPP. Thus, MMP-9 constitutes an endogenous islet protease that limits islet amyloid deposition and its toxic effects via degradation of hIAPP. Because islet MMP-9 mRNA levels are decreased in type 2 diabetic subjects, islet MMP-9 activity may also be decreased in human type 2 diabetes, thereby contributing to increased islet amyloid deposition and β-cell loss. Approaches to increase islet MMP-9 activity could reduce or prevent amyloid deposition and its toxic effects in type 2 diabetes.

A pathological characteristic of the islet in type 2 diabetes is the deposition of islet amyloid polypeptide (IAPP)4 as amyloid (1–3). In subjects with type 2 diabetes, the extent of amyloid is associated with both reduced β-cell mass (1–3) and increased β-cell apoptosis (3), suggesting that islet amyloid deposition is toxic to β-cells. IAPP is a normally soluble secretory product of pancreatic β-cells (5–8, 16, 17), elucidating mechanisms by which the aggregation of hIAPP into amyloid is toxic to β-cells (5–8). This β-cell loss occurs via activation of c-Jun N-terminal kinase (JNK) and downstream activation of both the intrinsic and extrinsic apoptosis pathways (16). Additionally, when hIAPP aggregation is inhibited by Congo red or overexpression of neprilysin, β-cell apoptosis is reduced, suggesting that hIAPP aggregation is an important mediator of β-cell toxicity (8, 16, 17).

Given the evidence that aggregation of hIAPP into amyloid is toxic to β-cells (5–8, 16, 17), elucidating mechanisms by which the aggregation of hIAPP is reduced or prevented could be beneficial for slowing or preventing β-cell loss and dysfunction in type 2 diabetes. These mechanisms could include reduction of hIAPP production and/or proteolytic degradation of hIAPP, the latter being the focus of this study.

4 The abbreviations used are: IAPP, islet amyloid polypeptide; Aβ, amyloid β; hIAPP, human IAPP; miAPP, mouse IAPP; MMP, matrix metalloproteinase.
Two enzymes have been implicated as playing a role in reducing hIAPP aggregation into amyloid, insulin-degrading enzyme (or insulysin) and neprilysin (17–19). Inhibition of insulin-degrading enzyme activity in RIN-m5F insulinoma cells treated with hIAPP resulted in increased amyloidogenesis and reduced cell viability (18). Similarly, inhibition of neprilysin in cultured hIAPP transgenic mouse islets increased islet amyloid formation and H9252-cell apoptosis (17), whereas increased neprilysin protected against amyloid formation and H9252-cell apoptosis (19).

Two other proteases, MMP-2 and MMP-9 (also known as gelatinase A and B), are each involved in reducing aggregation of another amyloidogenic peptide, amyloid β (Aβ) (20, 21), the unique constituent of brain amyloid in Alzheimer disease. Thus, MMP-2 and MMP-9 may have the potential to reduce hIAPP aggregation. Both of these MMPs are zinc-dependent metalloproteinases that are synthesized as inactive proenzymes and activated via proteolytic cleavage by other proteases upon release from the cell (22). Importantly in relation to islet amyloid, both MMP-2 and MMP-9 have been shown to be expressed in human islets (23), but their role in hIAPP aggregation is not known. Further, due to their extracellular location (24), they are in an ideal position to interact with hIAPP in the extracellular space where amyloid has been shown to occur (14). Thus, in the current study we sought to determine whether MMP-2 and MMP-9 play a role in reducing or limiting islet amyloid deposition.

EXPERIMENTAL PROCEDURES

Islet Isolation and Culture—Pancreatic islets were isolated from 10-week-old male and female hemizygous hIAPP transgenic mice or nontransgenic littermate controls on an F1 C57BL/6 × DBA/2 background (25, 26). The studies were approved by the Institutional Animal Care and Use Committee of the Veterans Affairs Puget Sound Health Care System.

Islets were handpicked and cultured overnight in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 11.1 mM glucose. They were subsequently cultured for an additional 7 days in RPMI 1640 medium containing 0.2% bovine serum albumin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 16.7 mM glucose with or without the global MMP inhibitor GM6001 (25 μM), MMP-9 inhibitor I (100 nM), or MMP-2 inhibitor III (100 nM) (EMD Biosciences, San Diego, CA). Media were changed every 48 h.

RNA Isolation and Quantitative Real Time PCR—Total RNA was isolated from 25 islets per condition (High Pure RNA isolation kit, Roche Applied Science) and reverse transcribed (High Capacity cDNA Archive kit, Applied Biosystems). MMP-2 and MMP-9 mRNA levels were measured in triplicate using the TaqMan system (ABI Prism 7000; Applied Biosystems) with assays on demand (MMP-2, Mm00439508_m1 and MMP-9, Mm00600163 _m1; Applied Biosystems), with 18 S rRNA levels as the endogenous control (Hs99999901_s1; Applied Biosystems). mRNA levels were expressed relative to 18 S using 2^−ΔCt and normalized to nontransgenic controls.

Zymography—Activity of MMP-2 and MMP-9 was determined via gelatinase zymography. Islet conditioned medium was concentrated using Icon™ concentrator spin columns (20,000 molecular weight cutoff; Pierce) then electrophoresed on a 10% gelatin Novex® Zymogram gel (Invitrogen). The gel...
was then incubated in Zymogram Renaturing Buffer and developed overnight in Zymogram Developing Buffer (Invitrogen), stained (0.5% (w/v) bromphenol blue, 10% (v/v) acetic acid and 50% (v/v) methanol), and counterstained (10% (v/v) acetic acid). Gelatinase activity was visualized as negatively stained bands. MMP-2 and MMP-9 were identified by molecular weight using recombinant MMP-2 and MMP-9 as markers. Bands were quantified and expressed relative to nontransgenic MMP-9 activity.

**MMP Assay**—The action of the MMP inhibitors (GM6001, MMP-9 inhibitor I, or MMP-2 inhibitor III at 25 μM, 100 nM, or 100 nM respectively) on the activity of purified recombinant MMP-1, -2, -3, -7, -9, and -14 (EMD Biosciences) was determined using the fluorometric SensoLyte™ 520 Generic MMP Assay kit (AnaSpec, San Jose, CA).

**Histological Measurements**—Islets were fixed for histology for 30 min in 4% (w/v) phosphate-buffered paraformaldehyde, embedded in agar and then in paraffin (25). Ten-μm sections were cut, and sections at 100-μm intervals throughout the islet pellet were stained with thioflavin S to visualize amyloid deposits and insulin antibody to visualize β-cells (25). Islet area and thioflavin S-positive area were determined using Image Pro Plus software (Media Cybernetics, Bethesda, MD), and amyloid severity (Σ thioflavin S area/Σ islet area × 100%) was calculated (26).

For determination of β-cell apoptosis, sections were stained with propidium iodide to visualize condensed and fragmented apoptotic nuclei and insulin antibody to visualize β-cells (8). The percentage of β-cells that were apoptotic was determined by manual counting of condensed nuclei in insulin-positive cells. An average of 25 islets per condition per experiment was analyzed with the observer blinded to the genotype and culture conditions of the islets.

**Mass Spectrometry**—50 μM hIAPP or mIAPP peptide (Bachem, Torrance, CA), the latter as a nonamyloidogenic control, was incubated with and without 50 nM purified recombinant enzyme (MMP-2, MMP-9, or MMP-7; Calbiochem) in buffer (phosphate-buffered saline containing 1 mM CaCl₂ and 0.5 mM MgCl₂) at 37 °C for 16 h and then snap frozen. Samples were desalted using C4 ZipTips (Millipore) and analyzed on a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (Voyager DE-STR, Applied Biosystems) with recrystallized α-cyano-4-hydroxycinnamic acid as the matrix. Spectra were collected from 100 shots from 500 to 5000 m/z. External calibration was performed using melitten (Sigma) as a calibration standard.

**Islet MMP-9 mRNA in Human Type 2 Diabetic and Control Subjects**—Human islet mRNA data from a published database (27) was kindly provided by Dr. Gordon Weir (Joslin Diabetes
MMP-9 mRNA data were analyzed from RNA extracted from β-cells in frozen pancreas specimens from four subjects with type 2 diabetes and four nondiabetic controls.

RESULTS

MMP-2 and MMP-9 mRNA and Activity in Islets—MMP-2 and MMP-9 mRNA were detectable in both nontransgenic and hIAPP transgenic mouse islets after 7 days of culture in 16.7 mM glucose (Fig. 1A), conditions where only hIAPP transgenic islets develop amyloid. Neither the presence of the hIAPP transgene nor islet amyloid affected MMP-2 or MMP-9 expression (Fig. 1A). In islet-conditioned medium, MMP-9 activity was detectable by zymography at comparable levels between hIAPP transgenic and nontransgenic islets (Fig. 1B). In contrast, MMP-2 activity was not detectable in islet-conditioned medium from mice of either genotype (Fig. 1B).

Specificity of MMP Inhibitors—To test whether MMP-2 and/or MMP-9 plays a role in reducing islet amyloid formation, the specificity/selectivity of several MMP inhibitors was first tested using a fluorometric MMP assay. The efficacy of each inhibitor to reduce the activity of both recombinant MMP-2 and MMP-9 and one member from each of the remaining MMP subfamilies was tested (Fig. 2). The broad spectrum MMP inhibitor GM6001 resulted in virtually complete inhibition of MMP-2 and MMP-9 activity as well as members from all other MMP subfamilies. The MMP-2 inhibitor III selectively inhibited MMP-2 activity, with no major effect upon MMP-9 or the other subfamilies. In contrast, the MMP-9 inhibitor I was effective against both MMP-2 and MMP-9, but not against the other MMP subfamilies.
MMPs from the remaining subfamilies. Because of its lack of specificity for MMP-9, this latter inhibitor is hereafter referred to as MMP-2/9 inhibitor. No other selective MMP-9 inhibitor was available.

**Effect of MMP Inhibitors on Islet Amyloid Formation and β-Cell Apoptosis**—Culture of hIAPP transgenic islets for 7 days in 16.7 mm glucose resulted in amyloid formation (Fig. 3, A and B). As expected, nontransgenic islets did not develop amyloid; amyloid formation in hIAPP transgenic islets was associated with increased β-cell apoptosis compared with nontransgenic islets (Fig. 3, C and D). Treatment with the broad spectrum MMP inhibitor GM6001 resulted in a significant increase in both amyloid deposition and β-cell apoptosis in hIAPP transgenic islets compared with hIAPP transgenic islets without inhibitor and nontransgenic islets with inhibitor (Fig. 3). Similarly, treatment of hIAPP transgenic islets with the MMP-2/9 inhibitor increased amyloid deposition and β-cell apoptosis compared with hIAPP transgenic islets without inhibitor and nontransgenic islets with inhibitor (Fig. 3). In contrast, treatment of hIAPP transgenic islets with the MMP-2 inhibitor had no effect on amyloid deposition or β-cell apoptosis (Fig. 3). There was no effect of any inhibitor to modulate β-cell apoptosis independent of islet amyloid as shown in nontransgenic islets (Fig. 3D).

**IAPP Degradation by MMP-2 and MMP-9**—Given that inhibiting MMP-9 activity increased amyloid formation, we sought to determine whether the mechanism by which the gelatinases (MMP-2 and MMP-9) normally limit/reduce islet amyloid deposition could be via hIAPP degradation. Thus, the mass spectrometry profile of hIAPP was analyzed after the peptide had been incubated with MMP-2 or MMP-9. When the hIAPP peptide was examined without exposure to either MMP-2 or MMP-9, the mass spectrometry profile showed a single peak at 3904 Da indicating intact, full-length hIAPP (Fig. 4A). Following incubation with MMP-2, hIAPP was cleaved as shown by a decrease in the intact 3904-Da peptide and an increase in smaller hIAPP fragments (Fig. 4B). After incubation with MMP-9, hIAPP was similarly cleaved with a decrease in the intact peptide and an increase in smaller hIAPP fragments (Fig. 4C). Incubation of hIAPP with MMP-7, as a nongelatinase control, had no effect to cleave the peptide (Fig. 4D).

To determine whether MMP-2 and MMP-9 degradation of IAPP was selective for amyloidogenic hIAPP, mIAPP was also incubated with MMP-2, MMP-9, and MMP-7. mIAPP alone showed a peak at ~3920 Da (Fig. 4E). Incubation with MMP-2, MMP-9, or MMP-7 did not cleave mIAPP as shown by single peaks at 3920 Da with no smaller fragments (Fig. 4, F–H).

**Islet MMP-9 mRNA in Human Type 2 Diabetes**—To determine whether islet MMP-9 is reduced in type 2 diabetes, and thus may explain the increased amyloid formation, using a published database (27), human islet MMP-9 mRNA levels were examined in subjects with and without type 2 diabetes. MMP-9 mRNA was decreased by 34% in type 2 diabetic islets compared with control islets (Fig. 5).

**DISCUSSION**

hIAPP is a normal product of the β-cell that is co-secreted with insulin in response to glucose and nonglucose stimuli (4). hIAPP has the propensity to aggregate and in so doing can induce β-cell apoptosis (5–8, 16, 17). Thus, it is logical that mechanisms would be in place in the islet to facilitate clearance and/or degradation of excess hIAPP, prevent its accumulation in the extracellular space, and thereby limit its toxicity. In the current study we have identified MMP-9 as an enzyme that plays a role in vitro to limit or reduce the aggregation of hIAPP into amyloid via degradation of hIAPP.

For MMP-2 or MMP-9 to be a viable candidate as an IAPP-degrading enzymes, it was critical to demonstrate their expression and activity in islets. MMP-9 was expressed and active in both hIAPP transgenic and nontransgenic islets, consistent with the description of its expression in human islets (23). MMP-2 mRNA was also present in mouse islets, but in contrast to MMP-9, MMP-2 activity was not detected in either hIAPP transgenic or nontransgenic mouse islets. This finding is consistent with previous reports showing MMP-2 activity to be only detectable for a short period during embryogenesis in both rat and mouse pancreas (28, 29), and importantly, a lack of detectable MMP-2 activity in islets of 6–9 week old ZDF rats (30). Therefore, it is likely that MMP-2 is only present at very low levels in the adult mouse islet and does not appreciably contribute to reducing amyloid formation.

Inhibition of MMPs with the broad spectrum MMP inhibitor GM6001 resulted in increases in amyloid formation and β-cell apoptosis. This indicates that one or more MMPs normally act to reduce the accumulation of hIAPP as amyloid and thereby also limit its toxic effects. Further, as the MMP-2/9 inhibitor increased amyloid formation to the same extent as with the broad spectrum MMP inhibitor, it would appear that the effect on amyloid formation seen with GM6001 was likely due to inhibition of MMP-2 and/or MMP-9. Importantly, we found that inhibition of MMP-2 alone had no effect on islet amyloid formation or β-cell apoptosis, consistent with the finding that no active MMP-2 is detectable. Thus, it appears likely that it is the inhibition of MMP-9 that is responsible for the effects seen on amyloid formation with both the broad spectrum MMP inhibitor and the MMP-2/9 inhibitor.
MMP-9 and Islet Amyloid

![Diagram of hIAPP and mIAPP cleavage sites](image)

**FIGURE 6.** Amino acid sequences of hIAPP and mIAPP showing putative site of cleavage by MMP-2 and MMP-9. The sequence required for amyloidogenesis in hIAPP is indicated. This region is nonamyloidogenic in mIAPP due to several amino acid substitutions, indicated with shading.

We and others have recently shown that neprilysin, a membrane metalloendopeptidase, is capable of reducing islet amyloid deposition (17, 19). Here we show that MMP-9 degrades hIAPP and reduces islet amyloid deposition. Further, although MMP-2 was not expressed at appreciable levels in islets, we found that MMP-2 could also cleave hIAPP and that the cleavage products matched those from MMP-9 digestion of hIAPP. This indicates that MMP-2 and MMP-9 likely cleave hIAPP at the same site(s). From the molecular weight of the digestion products, we determined the putative cleavage site to be between amino acids 25 (alanine) and 26 (isoleucine) of hIAPP (Fig. 6). It has been shown that MMP-2 and MMP-9 prefer a serine, glycine, or alanine immediately before the cleavage site and an aliphatic, hydrophobic residue (leucine or isoleucine) immediately after the cleavage site (31, 32). Thus, this putative cleavage site is consistent with the known preferred substrate residues for MMP-2 and MMP-9. This cleavage site is within the region of hIAPP shown to be critical for amyloid fibril formation (9) and the cleavage products hIAPP1–25 and hIAPP26–37 have themselves not been reported to be amyloidogenic (34–35). Therefore it is plausible that the peptides remaining after MMP-2 or MMP-9 cleavage would be unable to aggregate and form amyloid, potentially explaining how MMP-9 action may normally limit amyloid formation and its toxic effects. Further, this ability to cleave hIAPP was shown not to be a general characteristic of MMP enzymes, as MMP-7 was not able to cleave hIAPP. Interestingly, we also found that nonamyloidogenic mIAPP was not cleaved by MMP-2 or MMP-9, indicating that the amino acid differences between hIAPP and mIAPP are critical for the degrading action of MMP-2 and MMP-9. The sequence of the amyloidogenic region of hIAPP (amino acids 20–29) differs at several amino acids compared with mIAPP (Fig. 6). In fact, the amino acids at the putative cleavage site of hIAPP (amino acids 25 and 26, alanine and isoleucine) are both different in mIAPP (proline and valine). This amino acid sequence in mIAPP is not consistent with the preferred cleavage site residues for MMP-2 and MMP-9 action (31, 32), explaining why mIAPP is not cleaved by MMP-2 or MMP-9.

Our data suggest that in the islet, MMP-9, along with other proteases such as insulin-degrading enzyme (18) and neprilysin (17, 19), acts to reduce islet amyloid accumulation. However, despite this, islet amyloid deposition is present in the majority of patients with type 2 diabetes (3, 36). A possible reason for this is that the action of these enzymes is only able to limit the extent of amyloid formation and not prevent it altogether. Alternatively, regulation of MMP-9, and possibly these other proteases, may be dysfunctional in type 2 diabetes and that this is one of the reasons why amyloid deposition essentially only occurs in diabetic subjects. In fact, in human samples islet MMP-9 mRNA levels were decreased by 34% in type 2 diabetic islets compared with control islets. Thus, down-regulation of islet MMP-9 may be contributing to islet amyloid deposition and its associated β-cell loss in type 2 diabetes.

In summary, our findings suggest that MMP-9 can reduce amyloid deposition in the islet by degrading hIAPP. Further, islet MMP-9 mRNA is decreased in subjects with type 2 diabetes and thus may contribute to amyloid formation and the exacerbation of β-cell dysfunction and death. Thus, interventions aimed at increasing MMP-9 in the islet could potentially reduce or prevent hIAPP aggregation, thereby improving β-cell survival.

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