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INHIBITION OF GLYCOSAMINOGLYCAN SYNTHESIS AND PROTEIN GLYCOSYLATION WITH WAS-406 AND AZASERINE RESULT IN REDUCED ISLET AMYLOID FORMATION IN VITRO

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SUMMARY

Deposition of islet amyloid polypeptide (IAPP) as amyloid in the pancreatic islet occurs in ~90% of individuals with type 2 diabetes and is associated with decreased islet β -cell mass and function. Human IAPP (hIAPP), but not rodent IAPP, is amyloidogenic and toxic to islet β cells. In addition to IAPP, islet amyloid deposits contain other components, including heparan sulfate proteoglycans (HSPGs). The small molecule 2-acetamido-1,3,6-tri-*O*-acetyl-2,4-dideoxy- α -D-xylo-hexopyranose (WAS-406) inhibits HSPG synthesis in hepatocytes and blocks systemic amyloid A deposition *in vivo*.

To determine whether WAS-406 inhibits localized amyloid formation in the islet, we incubated hIAPP transgenic mouse islets for up to seven days in 16.7 mM glucose (conditions that result in amyloid deposition), plus increasing concentrations of the inhibitor. WAS-406, at doses of 0, 10, 100 and 1000 μ M resulted in a dose-dependent decrease in amyloid deposition (% islet area occupied by amyloid: 0.66 ± 0.14 , 0.10 ± 0.06 , 0.09 ± 0.07 and $0.004 \pm 0.003\%$, $p < 0.001$) and an increase in β -cell area in hIAPP transgenic islets (55.0 ± 2.6 vs. $60.6 \pm 2.2\%$ islet area for 0 vs. 100 μ M inhibitor, $p = 0.05$). Glycosaminoglycan, including heparan sulfate, synthesis was inhibited in both hIAPP transgenic and non-transgenic islets (the latter a control that do not develop amyloid), while O-linked protein glycosylation was also decreased, and WAS-406 treatment tended to decrease islet viability in non-transgenic islets. Azaserine, an inhibitor of the rate limiting step of the hexosamine biosynthesis pathway, replicated the effects of WAS-406, resulting in reduction of O-linked protein glycosylation and glycosaminoglycan synthesis and inhibition of islet amyloid formation. In summary, interventions that decrease

both glycosaminoglycan synthesis and O-linked protein glycosylation are effective in reducing islet amyloid formation, but their utility as pharmacological agents may be limited due to adverse effects on the islet.

Islet amyloid deposition is a pathogenic hallmark of the islet in type 2 diabetes, occurring in the vast majority of individuals with the disease (33), and is associated with decreased β -cell mass and function (5,32). The unique amyloidogenic component of islet amyloid is the β -cell peptide islet amyloid polypeptide (IAPP, amylin) (6,35). Human IAPP (hIAPP) is amyloidogenic and *in vitro* studies have shown that early aggregates or oligomers of hIAPP are cytotoxic, leading to β -cell death via apoptosis (12,22). In contrast, the rodent (rat and mouse) forms of IAPP differ from hIAPP in a number of critical amino acids, rendering rodent IAPP non-amyloidogenic and non-toxic (34). Due to these species-specific differences, several groups have produced transgenic mice expressing hIAPP in their pancreatic islet β -cells in order to create models of islet amyloid deposition. In our colony of hIAPP transgenic mice, male mice develop islet amyloid deposits *in vivo* following one year of high fat feeding (29). We have also recently developed a rapid *in vitro* model of islet amyloid deposition by culturing isolated islets from our hIAPP transgenic mice in high glucose for seven days (10,38).

Besides the amyloidogenic peptide IAPP, islet amyloid contains other components that are common to all amyloidoses, including those formed in Alzheimer's disease (A β amyloid) and chronic inflammation (AA amyloid). These include apolipoprotein E (4), serum amyloid P component (24) and heparan sulfate proteoglycans (HSPGs) (36), all of which may contribute to hIAPP amyloidogenesis and its related cytotoxicity.

HSPGs in particular may play a role in islet amyloidogenesis. The HSPG perlecan has been shown to be present in human β cells from individuals with and without type 2 diabetes (13) and the β cell synthesizes several HSPGs that are capable of binding amyloidogenic hIAPP but not non-amyloidogenic rodent IAPP (25). Further, binding of amyloidogenic peptides, including IAPP, to HSPGs via their heparan sulfate (HS) glycosaminoglycan (GAG) chains has been shown to stimulate amyloid fibril formation (2,3). Thus, HSPGs may play a critical role in islet amyloid formation and evidence that decreasing GAG synthesis reduces islet amyloid formation would provide further evidence to support this hypothesis.

We have generated a series of *N*-acetylglucosamine analogs that act as small molecule inhibitors of GAG synthesis (17-20). These compounds are effective in reducing amyloid formation in a mouse model of AA amyloidosis (20), and in a transgenic mouse model of CNS A β amyloid (16). In the present study we examined the effect of one of these compounds, 2-acetamido-1,3,6-tri-*O*-acetyl-2,4-dideoxy- α -D-xylo-hexopyranose (WAS-406), on β -cell GAG synthesis and on islet amyloid formation *in vitro*.

Precursors for GAG synthesis are synthesized via the hexosamine biosynthesis pathway (HBP). The HBP is a nutrient sensing pathway that has many additional effects in the cell including regulation of O-linked protein glycosylation. Azaserine, an inhibitor of glutamine:fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme of the HBP, has previously been shown to have no effect on GAG synthesis in arterial smooth muscle cells (28). Therefore, we compared the effects of azaserine and WAS-406 on GAG synthesis and islet amyloid formation *in vitro*.

RESEARCH DESIGN AND METHODS

Isolation and Culture of Mouse Islets

Hemizygous hIAPP transgenic and non-transgenic mice on a F1 C57BL/6 $\times$ DBA/2 background were bred and utilized under protocols approved by the Institutional Animal Care

and Use Committee of the VA Puget Sound Health Care System. Eight to ten-week old male and female mice were anesthetized with sodium pentobarbital (100 mg/kg i.p.), and pancreatic islets were isolated by collagenase digestion (Collagenase P, 0.5 mg/ml, Roche Applied Science, Indianapolis, IN) via bile duct cannulation, followed by pancreas excision. Islets were purified on a Histopaque gradient, hand picked and cultured overnight in RPMI-1640 containing 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 11.1 mM glucose. Islets were then cultured for up to seven days in medium containing 16.7 mM glucose alone or together with WAS-406 (10 - 1000 µM for dose response studies, 100 µM thereafter). In a subset of experiments, islets were also cultured in the presence of the HBP inhibitor azaserine (20 µM). Stock solutions of WAS-406 (100 mM) and azaserine (20 mM) were prepared in sterile water, and then diluted 1:1000 in culture medium. Culture medium was changed, and thus new compound was supplied to islets, every 48 hours.

Histological Determination of Islet Amyloid and β -cell Area

At the end of each experiment, islets were fixed in 4% (w/v) phosphate-buffered paraformaldehyde. Islets were embedded in agar, re-fixed in 4% paraformaldehyde, embedded in paraffin and processed for histology. Five-µm sections were cut throughout the islet pellet and sections at 100 µm intervals were stained for amyloid with thioflavin S and for insulin as previously described (9). Islet area was determined by circumscribing each islet image with a video cursor; the outline of islets is clearly visible when viewed in the thioflavin S channel. Islet-, thioflavin S-positive- and insulin-positive areas were determined for each islet cross-section in an average of 25 islets per experimental condition. From these data, the following measures were determined: islet amyloid prevalence (% islets containing thioflavin S positive staining), islet amyloid severity (Σ thioflavin S area / Σ islet area $\times$ 100%) and β -cell area (Σ insulin area / Σ islet area $\times$ 100%).

Insulin Secretion and Content

Insulin secretion was determined following seven days of culture. Islets (100-150 per condition) were loaded into a perfusion chamber and were pre-incubated in Krebs-Ringer bicarbonate buffer containing basal (1.67 mM) glucose for one hour, followed by measurement of basal insulin secretion at 1.67 mM glucose every 2 minutes for 8 minutes. Glucose-stimulated insulin secretion was then assessed by perfusion of islets with Krebs-Ringer bicarbonate buffer containing 16.7 mM glucose for 30 minutes (fractions collected every 2-5 minutes). Glucose-stimulated insulin secretion was expressed as the incremental area under the curve. Secretion data from these studies using only the hIAPP transgenic and non-transgenic islets cultured for seven days in 16.7 mM alone have been previously published (38).

For determination of insulin content islets were solubilized in acid ethanol (0.2 M HCl, 48 % (v/v) ethanol). Total protein was determined (Coomassie Plus Protein Assay; Pierce Biotechnology, Rockford, IL) and insulin content was measured by radioimmunoassay as described previously (10).

Islet Cell Viability

Islet cell viability after seven days of culture was assessed using the Cell Proliferation Kit I (Roche) according to the manufacturer's instructions. Twenty islets/well (triplicates for each condition) were incubated for four hours in culture medium containing 0.5mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Islets were solubilized by overnight incubation in solubilization buffer and solubilized formazan was quantified spectrophotometrically (590 nm).

Assessment of Glycosaminoglycan Synthesis in Islets and β -TC3 cells

Islets were isolated from hIAPP transgenic and non-transgenic mice and recovered overnight as described above. The immortalized islet β -cell line β -TC3 was plated at 1.2×10^6 cells/ml and cultured for 5 days prior to study in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin and 5.5 mM glucose. Islets (450 per plate) and β -TC3 cells were then metabolically labeled for 48 hours with [35 S]-Na₂SO₄ (100 μ Ci/ml; MP Biomedicals, Costa Mesa, CA) in medium containing 16.7 mM glucose alone or together with WAS-406 (100 μ M for islets and 10 - 1000 μ M for β -TC3 cells) or azaserine (20 μ M; in a subset of experiments). This approach labels newly synthesized GAG chains on proteoglycans.

Incorporation of [35 S]-Na₂SO₄ into GAGs was assessed in combined medium + cell preparations by cetylpyridinium chloride precipitation (31). Labeled media were collected in the presence of protease inhibitors (5 mM benzamidine, 100 mM 6-aminohexanoic acid, and 0.1 mM phenylmethylsulfonyl fluoride) and labeled islets/cells were solubilized in 8 M urea buffer (8 M urea, 2 mM EDTA, 0.25 M NaCl, 50 mM Tris-HCl, and 0.5% Triton-X 100 detergent, pH 7.4) containing protease inhibitors.

To determine the effects of WAS-406 to reduce GAG synthesis and its specificity for HS versus chondroitin/dermatan sulfate (CS/DS), labeled glycosaminoglycans were purified from β -TC3 cells. [35 S]-Na₂SO₄ labeling was performed as described above and cells solubilized in phosphate-buffered saline (PBS, pH 7.2) containing 1% (v/v) Triton X-100, pooled together with medium and incubated with pronase (*Streptomyces griseus* 100 μ g/ml, Roche) overnight at 37°C. GAGs were isolated by application to DEAE Sephacel equilibrated in PBS and eluted over a 0.15 - 0.8 M sodium chloride gradient. Residual core protein fragments were removed by alkaline elimination and borohydride reduction and the resulting glycosaminoglycan preparations digested with no enzyme (control), heparinase I, II + III (0.8, 0.4 and 0.8 U per digestion respectively, Sigma St. Louis, MO), chondroitinase ABC (0.03 U per digestion, Seikagaku USA, Cape Cod, MA) or a combination of heparinase I, II, III + chondroitinase ABC for three hours at 37°C. Reaction products were analyzed by molecular sieve chromatography (Sephacel CL-6B, equilibrated in 0.2 M Tris, 0.2 M NaCl, pH 7.0).

Western Blotting

Islet lysates (75 islets per culture condition) were prepared by sonication on ice in 20 mM Tris-HCl, 150 mM NaCl, 1% (v/v) Nonidet P-40, pH 7.5 followed by centrifugation for 20 minutes at 12,000g. Total protein concentration was determined using the Coomassie Plus Protein Assay (Pierce Biotechnology) and from this equal protein (15 μ g) was loaded for each sample. Samples were separated by SDS-PAGE, transferred to polyvinylidene fluoride membrane and non-specific binding blocked by incubation in 50 mM Tris-HCl, 150 mM NaCl, 0.1% (v/v) Tween-20 and 5% (w/v) non-fat dry milk, pH 7.5. Membranes were probed with antisera directed against O-linked *N*-acetylglucosamine (RL2, Affinity BioReagents, Golden, CO; 1:100 dilution). Primary antibody binding was detected using peroxidase-conjugated anti-mouse IgGs followed by enhanced chemiluminescence (PerkinElmer, Wellesley, MA).

Data Analysis

Data are expressed as mean $\pm$ SEM. For MTT islet viability, data are presented as % of non-transgenic islets cultured in 16.7 mM glucose due to variability among experiments resulting from the use of different lots of MTT kits. For metabolic labeling experiments $n=2-3$ proteoglycan preparations; for all other experiments $n=3-8$ islet preparations. Dose response data were compared by analysis of variance with Bonferroni post-hoc analysis. Two group comparisons were made by T-test or Mann Whitney U non-parametric test if the data were not normally distributed. A $p \leq 0.05$ was considered statistically significant.

RESULTS

Dose-Dependent Inhibition of Islet Amyloid Formation by WAS-406

Incubation of isolated hIAPP transgenic islets for seven days in 16.7 mM glucose and increasing concentrations of WAS-406 was associated with a marked, dose-dependent decrease in both the prevalence (Figure 1A) and severity (Figure 1B) of islet amyloid formation.

Effect of WAS-406 on β -cell Area, Insulin Secretion, Insulin Content and Islet Viability

Based on the dose-response study, a dose of 100 μ M of WAS-406 was selected for further assessments of islet morphology. The reduction in amyloid formation observed in hIAPP transgenic islets in the presence of WAS-406 was associated with an increase in β -cell area as a proportion of islet area (Figure 2A). In contrast, the proportion of islet area comprised of β -cells was not significantly altered in non-transgenic islets cultured in the presence or absence of 100 μ M WAS-406 (Figure 2A). Islet area did not differ among treatment conditions or between hIAPP transgenic and non-transgenic islets (data not shown). Islet insulin content was not different in hIAPP transgenic islets compared to non-transgenic islets cultured in 16.7 mM glucose alone (Figure 2B). However, WAS-406 treatment resulted in a significant increase in insulin content in both hIAPP transgenic and non-transgenic islets (Figure 2B). Glucose-stimulated insulin secretion ($n=4$ per condition) was not significantly different with WAS-406 treatment in hIAPP transgenic islets (11.1 ± 2.8 vs. 18.0 ± 6.9 μ U/islet.30 minutes for untreated vs. WAS-406) or non-transgenic islets (15.2 ± 1.3 vs. 22.2 ± 3.2 μ U/islet.30 minutes for untreated vs. WAS-406, $p=0.1$). Islet viability was significantly lower in hIAPP transgenic islets compared to non-transgenic islets cultured in 16.7 mM glucose alone (Figure 2C) as we have previously shown (38), and WAS-406 treatment was associated with a tendency towards a reduction in viability in non-transgenic islets ($p=0.065$) but no change in viability in hIAPP transgenic islets.

WAS-406 Decreases Glycosaminoglycan Synthesis in β -TC3 Cells and Islets

WAS-406 decreased incorporation of [35 S]-sulfate into newly synthesized GAGs in a dose-dependent manner in immortalized β -TC3 cells (Figure 3A). GAG synthesis was reduced by 62% in β -TC3 cells incubated with 100 μ M WAS-406 ($p<0.001$ vs. untreated cells). GAG synthesis during a 48-hour label was similarly reduced with WAS-406 (100 μ M) by 66% in both hIAPP transgenic and non-transgenic islets (Figure 3B). Ion exchange chromatography showed that β -TC3 cell GAGs synthesized in the presence and absence of WAS-406 eluted from DEAE Sephacel at a similar salt concentration (0.45 M NaCl; Figure 3C), although, as expected, the abundance of GAGs synthesized in the presence of WAS-406 was much reduced compared to untreated cells.

WAS-406 Inhibits Heparan and Chondroitin/Dermatan Sulfate Synthesis in β -TC3 Cells

To determine whether WAS-406 was specific for inhibition of heparan sulfate synthesis, equal numbers of counts of 35 S-sulfate-labeled GAGs synthesized by β -TC3 cells in the presence or absence of WAS-406 were digested with no enzyme (intact GAG control), heparinases I, II and III (to digest HS), chondroitinase ABC (to digest CS/DS) or the combination of heparinases I, II, III + chondroitinase ABC. If WAS-406 were specific for inhibition of HS synthesis, the residual GAGs synthesized in the presence of this compound would be expected to be insensitive to heparinase digestion, but sensitive to chondroitinase ABC treatment.

Analysis of reaction products by CL-6B Sepharose size exclusion chromatography showed that intact GAGs were of a similar size in control (Figure 4A) and WAS-406 (Figure 4E)-treated β -TC3 cells. As we showed previously, β -TC3 cells synthesize predominantly HS (25); thus a greater proportion of GAGs synthesized in control cells (without WAS-406) were

sensitive to heparinase I, II and III digestion (Figure 4B; shaded area denotes digested material, in this case heparinase-sensitive material) than to chondroitinase ABC digestion (Figure 4C). As expected, no intact GAGs were present following digestion with the combination of heparinase I, II and III and chondroitinase ABC (Figure 4D). β -TC3 GAGs synthesized in the presence of WAS-406 contained a similar proportion of heparinase I, II and III sensitive material (Figure 4F) and chondroitinase ABC sensitive material (Figure 4G) compared to control cells, and all material was sensitive to the combination of heparinase I, II and III and chondroitinase ABC treatment (Figure 4H).

WAS-406 Decreases O-linked Protein Glycosylation

Since WAS-406 is an analog of *N*-acetylglucosamine, an intermediate of the HBP, we analyzed flux through the HBP by measuring O-linked protein glycosylation in islets treated for seven days with 16.7 mM glucose alone or with WAS-406 (100 μ M) or the HBP inhibitor azaserine (20 μ M) as a positive control. WAS-406 and azaserine treatment in both non-transgenic islets (Figure 5) and hIAPP transgenic islets (not shown) resulted in a decrease in O-linked protein glycosylation as demonstrated by a reduced number of glycosylated proteins detected using an anti-O-linked *N*-acetylglucosamine antibody. The magnitude of the effect to decrease O-linked protein glycosylation was greater with WAS-406 than with azaserine.

Effect of the HBP Inhibitor Azaserine on Proteoglycan Synthesis and Islet Amyloid Deposition

Given the similar effects of WAS-406 and azaserine to decrease O-linked protein glycosylation, but the reported lack of an effect of azaserine to reduce GAG synthesis in arterial smooth muscle cells (28), we sought to determine the effect of azaserine on β -cell GAG synthesis and on islet amyloid formation. Unexpectedly, azaserine treatment in β -TC3 cells was associated with a significant reduction in GAG synthesis as determined by cetylpyridinium chloride precipitation (Figure 6A) and elution from DEAE Sephacel over a 0.15-0.8 M NaCl gradient (Figure 6B). Furthermore, incubation of hIAPP transgenic islets for seven days in 16.7 mM glucose and in the presence of azaserine (20 μ M) was associated with a marked reduction in islet amyloid prevalence (78 ± 5 vs 10 ± 6 % in untreated vs treated islets, $p < 0.01$) and islet amyloid severity (Figure 6C) and with an increase in the proportion of β -cell area to islet area (Figure 6D).

DISCUSSION

We have shown, for the first time that interventions that decrease GAG synthesis and protein glycosylation in islets are capable of inhibiting islet amyloid deposition and preventing the amyloid-induced loss of β -cell area *in vitro*. In the present study, WAS-406 and azaserine reduced both GAG synthesis and flux through the HBP, measured as O-linked protein glycosylation, resulting in a marked decrease in islet amyloid deposition.

While WAS-406 has been shown to be effective in reducing other amyloidoses (16,20), it was not clear that the same would hold true for islet amyloid formation. In the present study, we found that incubation of hIAPP transgenic islets with this compound resulted in a marked, dose-dependent decrease in both the prevalence and severity of islet amyloid; and this was associated with an increase in β -cell area. We also found that WAS-406 was effective in reducing [35 S]-sulfate incorporation into GAGs synthesized both by intact primary islets and by immortalized β -TC3 cells, in line with previous data that this compound also decreased GAG synthesis in hepatocytes; reducing both [35 S]-sulfate and [3 H]-glucosamine incorporation into glycosaminoglycans (20). In β -TC3 cells, residual GAGs synthesized in the presence of WAS-406 were sensitive to both heparinase and chondroitinase treatment, with the proportions of HS and CS/DS being similar to control cells. These data suggest that

WAS-406 is not specific for HS synthesis, which is not unexpected, given that the CS/DS disaccharide constituent *N*-acetylgalactosamine is formed by the epimerization of *N*-acetylglucosamine (a component of the repeating disaccharide in HS) and that levels of these two compounds exist in equilibrium, such that any intervention that perturbs the levels of one compound would also be expected to affect levels of the other. Since HS and CS/DS are important components of the cell and extracellular matrix, it is possible that the effect of decreased GAG synthesis with WAS-406 may have confounded the results of the present study. The inclusion of non-transgenic islets treated with WAS-406 allowed us to distinguish the effects of these compounds to decrease amyloid formation from other effects due to decreased GAG synthesis, but the potential adverse effects of long term use of WAS-406 may limit its utility as a pharmacological agent, as described in more detail below.

Previously, we have shown that a radiolabeled analog of *N*-acetylglucosamine, similar to WAS-406, can be incorporated into growing GAG chains, blocking their extension (20). It is possible that WAS-406 acts via this mechanism in β cells, although in the present study we did not evaluate whether it was directly incorporated into GAGs. Alternatively, WAS-406 may reduce islet GAG synthesis via down-regulation of the HBP, the biosynthetic pathway responsible for *N*-acetylglucosamine and *N*-acetylgalactosamine synthesis. The observed marked decrease in O-linked protein glycosylation following incubation of islets for seven days with WAS-406 is consistent with down-regulation of the HBP.

Unexpectedly, incubation of islets with azaserine also resulted in a reduction of GAG synthesis in β -TC3 cells. This differs from a previous study showing no effect of azaserine in the presence of high glucose to alter GAG synthesis in arterial smooth muscle cells (28). This may be due to the fact that, in that cell type, substrate supply through the HBP was not rate limiting for GAG synthesis at high glucose. However, increasing glucose concentrations have been associated with increased HBP activity in other cell types (7) and this is likely also the case in β cells, since β -cell glucose metabolism is regulated by the high K_m enzyme glucokinase. Thus, as our data suggest, azaserine would be predicted to be more effective at inhibiting GAG synthesis at high glucose in β cells than in smooth muscle cells. The decreases in GAG synthesis and O-linked protein glycosylation with azaserine were smaller in magnitude than those seen with WAS-406, which is likely a result of the lower dose of azaserine (20 μ M vs. 100 μ M for WAS-406). This lower dose of azaserine was chosen based on previous studies in mouse islets (37), with a higher dose being avoided due to concerns of toxicity. However, this lower dose of azaserine was extremely effective in inhibiting islet amyloid formation, suggesting that modulation of flux through the HBP is important for islet amyloid formation.

While it is possible that the effects of WAS-406 and azaserine to downregulate HBP flux in general, rather than a specific effect on GAG synthesis, may explain their ability to reduce islet amyloid formation, other lines of evidence support our hypothesis that decreased GAG, and particularly HS, synthesis is critical for the deposition of amyloid. First, several *in vitro* studies have shown that the HSPG perlecan, HS and the related glycosaminoglycan heparin can bind amyloidogenic peptides including hIAPP (2,3,25) and that this interaction leads to increased amyloid fibril formation (2,3). HS is more effective in increasing hIAPP fibril formation than CS/DS (2), suggesting a specific effect for HS in fibril formation. More direct evidence comes from studies using a murine model of AA amyloidosis together with over-expression of the enzyme heparanase that results in *in vivo* fragmentation of HS, but does not target CS/DS (21). Transgenic mice overexpressing heparanase showed marked resistance to hepatic and renal amyloid deposition but developed amyloid deposition in the spleen in a model of AA amyloidosis. The differential organ sensitivity in this transgenic mouse was inversely correlated with the organ expression of the transgene. That is, the heparanase transgene and its protein were expressed in kidney and liver but not the spleen. Thus, in the same animal fragmentation of HS was observed in kidney and liver but not the spleen rendering only the

spleen sensitive to AA amyloid deposition. This argues for a clear role of HS in amyloidogenesis.

Our observation that decreasing glycosaminoglycan synthesis with an *N*-acetylglucosamine analog results in reduced islet amyloid formation is in keeping with findings that these compounds reduce amyloid formation in mouse models of AA and CNS amyloidosis (16,20). However, while these effects were similar and amyloid deposits derived from different amyloidogenic peptides contain several invariant components, it is important to recognize that the role of these components in amyloid formation is not equivalent among different amyloidoses. For example, apolipoprotein E is important in the development of A β amyloid, as shown by delayed and reduced amyloid deposition in a mouse model of A β deposition lacking one or both apolipoprotein E alleles (1). In contrast, we found that while apolipoprotein E is a component of islet amyloid, apolipoprotein E deficiency had no effect on islet amyloid formation in human IAPP transgenic mice (30). Further, we found that apolipoprotein E is not synthesized by islet β -cells (30), suggesting that it may be trapped during the deposition of islet amyloid, perhaps by binding to proteoglycans, but that it appears to have no causative role in islet amyloidogenesis.

While we have observed that WAS-406 and azaserine are capable of decreasing amyloid formation, an important aspect of our findings is that they highlight the need for specificity in the development of amyloid inhibitors. While increased flux through the HBP has been shown to be detrimental to the β cell (14,27), protein glycosylation is critical for normal cellular function, such that decreased protein glycosylation is associated with impaired insulin secretion (37). Thus, the effects of chronic down-regulation of the HBP by WAS-406 or azaserine may have effects on the cell that are unrelated to their ability to reduce islet amyloid formation and may be detrimental to the β cell. Islet viability tended to be decreased in non-transgenic islets treated with WAS-406, and islet viability did not improve when islet amyloid was inhibited with WAS-406 in contrast to our findings with other amyloid inhibitors (Zraika S et al, unpublished observation), consistent with a detrimental effect of WAS-406 on islet viability. In contrast, insulin content was significantly increased in both hIAPP transgenic and non-transgenic islets with WAS-406 treatment, a finding seemingly at odds with the viability data. However, glucose-stimulated insulin secretion was not significantly altered (in islets from either genotype) in the presence of WAS-406, despite this significant increase in insulin content. Thus, we speculate that the increase in insulin content may occur, at least in part, due to a failure to adequately increase insulin secretion following WAS-406 treatment, consistent with a role of WAS-406 to decrease β -cell secretory function and impair islet viability.

Even in the absence of down-regulation of the HBP, systemic down-regulation of GAG synthesis would also be expected to have detrimental effects on the cell, as GAGs at the cell surface and in the extracellular matrix are important for sequestration and signaling of growth factors and chemokines (11). Thus, alternative approaches to reduce islet amyloid deposition have to be pursued. To date these have mainly targeted the amyloidogenic peptide IAPP. Several groups have described peptide-based inhibitors that are effective at reducing fibril formation and/or cytotoxicity of synthetic hIAPP (15,26). However, the bioavailability and thus applicability of these compounds have not been studied in models of *de novo* islet amyloid formation. Similarly, small molecules such as Congo red and rifampicin have also been shown to reduce hIAPP fibril formation (8,23), but further work is required to investigate their toxicity and/or efficacy in long-term studies.

In summary, our data demonstrate that small molecules such as WAS-406 and azaserine are highly effective inhibitors of islet amyloid formation. However, further studies with more specific interventions will be required to definitively prove that HS is required for islet amyloid deposition. Understanding of the actions of compounds that are capable of inhibiting islet

amyloid will be useful in developing therapeutic interventions to reduce amyloid formation that occurs as part of the islet lesion in type 2 diabetes.

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Abbreviations

CS, chondroitin sulfate
 DS, dermatan sulfate
 DEAE, diethylaminoethyl
 GAG, glycosaminoglycan
 hIAPP, human islet amyloid polypeptide
 HBP, hexosamine biosynthesis pathway
 HS, heparan sulfate
 HSPG, heparan sulfate proteoglycan
 IAPP, islet amyloid polypeptide
 MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
 WAS-406, 2-acetamido-1,3,6-tri-*O*-acetyl-2,4-dideoxy- α -D-xylo-hexopyranose

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Figure 1.

Dose-dependent decrease in islet amyloid prevalence (% islets with amyloid; panel A) and severity (% islet area occupied by amyloid; panel B) in hIAPP transgenic islets cultured for 7 days in the presence of increasing concentrations of WAS-406. n=4-8 per condition. * $p < 0.05$ or # $p < 0.005$ vs. untreated islets.

Figure 2.

Incubation of hIAPP transgenic (hIAPP) islets for seven days in the presence of 100 μ M WAS-406 resulted in a significant increase in β -cell area as a proportion of islet area compared to hIAPP transgenic islets cultured in the absence of WAS-406 (panel A). β -cell area in non-transgenic (NT) islets was not significantly affected by WAS-406 (panel A). Islet insulin content was significantly increased in both hIAPP transgenic and non-transgenic islets cultured for seven days in the presence of WAS-406 (panel B). Islet viability was significantly lower in hIAPP transgenic islets than in non-transgenic islets cultured for seven days in the absence of WAS-406 (panel C). WAS-406 tended to decrease islet viability in non-transgenic islets, and had no effect on islet viability in hIAPP transgenic islets. $n=5-8$ per condition. * $p \leq 0.05$ vs. untreated hIAPP transgenic islets; # $p \leq 0.01$ vs. untreated non-transgenic islets.

Figure 3.

Analysis of glycosaminoglycan synthesis during a 48-hour incubation with WAS-406. Incorporation of [^{35}S]-sulfate into newly synthesized GAGs was assessed by cetylpyridinium chloride precipitation (panels A and B). WAS-406 treatment resulted in a dose-dependent decrease in GAG synthesis in β -TC3 cells (panel A) and a decrease in GAG synthesis in both hIAPP transgenic and non-transgenic (NT) islets (panel B). $n=2-3$ preparations per condition. [^{35}S]-sulfate-labeled GAGs synthesized over a 48-hour period by β -TC3 cells in the absence (open circles) and presence (closed triangles) of 100 μM WAS-406 and eluted from DEAE Sephacel over a 0.15-0.8 M NaCl gradient (panel C). The abundance of GAGs was decreased in the presence of WAS-406 but the NaCl concentration at which these GAGs eluted from DEAE Sephacel was not different between GAGs synthesized in the presence or absence of WAS-406. * $p<0.05$ vs. untreated cells/islets.

Figure 4.

Sepharose CL-6B size-exclusion analysis of [^{35}S]-sulfate-labeled β -TC3 GAGs synthesized in 16.7 mM glucose alone (panels A-D) or together with WAS-406 (100 μM , panels E-H). GAGs were treated with no enzyme (panels A and E), heparinase I, II and III (panels B and F), chondroitinase ABC (panels C and G) or heparinase I, II and III + chondroitinase ABC (panels D and H). A right-shift in the size exclusion profile is indicative of sensitivity to a given enzyme combination (shaded areas correspond to digested material). A greater proportion of β -TC3 GAGs were sensitive to heparinase (panel B) than chondroitinase (panel C), with all material being sensitive to digestion with a combination of heparinase + chondroitinase (panel D). GAGs synthesized in the presence of WAS-406 showed similar sensitivity to heparinase

(panel F), chondroitinase (panel G) and the combination (panel H), demonstrating that WAS-406 does not disproportionately alter HS or CS/DS synthesis.

Figure 5.

Western blot showing O-linked protein glycosylation in non-transgenic islets cultured for seven days in the presence or absence of 100 μ M WAS-406 or 20 μ M azaserine. Several O-linked *N*-acetylglucosamine-modified proteins are detected in islets following seven days of culture in 16.7 mM glucose. In contrast, a decrease in O-linked *N*-acetylglucosamine-modified proteins was seen following WAS-406 or azaserine treatment, consistent with a down-regulation of flux through the hexosamine biosynthesis pathway (HBP).

Figure 6.

Analysis of glycosaminoglycan synthesis in β -TC3 cells during a 48-hour incubation with or without azaserine (20 μ M). Azaserine reduced incorporation of [35 S]-sulfate into newly synthesized proteoglycans from β -TC3 cells, assessed by cetylpyridinium chloride precipitation (panel A), and was associated with a reduction in abundance of GAGs assessed by elution from DEAE Sephacel over a 0.15-0.8 M NaCl gradient (panel B; untreated cells shown in open circles, 20 μ M azaserine shown in closed squares). n=3 per condition *p<0.05 vs. untreated cells.

Incubation of hIAPP transgenic islets for 7 days in 16.7 mM glucose and 20 μ M azaserine was associated with a significant reduction in islet amyloid severity (panel C) and a significant increase in β -cell area (panel D). n=4 per condition. # $p \leq 0.01$ vs. untreated islets.