ORIGINAL ARTICLE

Anti-aging Glycopeptide Protects Human Islets Against Tacrolimus-related Injury and Facilitates Engraftment in Mice

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Running title: AAGP protects transplanted human islets
Keywords: Islet transplantation, hypoxia, ischemia-reperfusion, islet loss, islet engraftment.

Abbreviations: Antifreeze proteins (AFP), Antiaging Glycopeptide (AAGP), Post-transplant Diabetes Mellitus (PTDM), Islet Equivalent (IEQ), Static Glucose-Stimulated Insulin Secretion (s-GSIS), Dynamic Islet Perfusion (D-IP), Reactive Oxygen Species (ROS), Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), Streptozotocin (STZ), Intraperitoneal Glucose-Tolerance Test (IPGTT), Standard Error of the Mean (SEM), Tacrolimus (Tac), Calcineurin Inhibitor (CNI), and Keratinocyte Chemokine (KC), Intracellular calcium concentration ([Ca$^{2+}$]$_i$)
ABSTRACT

Clinical islet transplantation has become an established treatment modality for selected patients with Type 1 diabetes. However, a large proportion of transplanted islets is lost through multiple factors including immunosuppressant-related toxicity, often requiring more than one donor to achieve insulin independence. Based on the cytoprotective capabilities of antifreeze proteins (AFP) we hypothesize that supplementation of islets with synthetic AFP analogue, Anti-aging Glycopeptide (AAGP) would enhance post-transplant engraftment and function, and would protect against tacrolimus (Tac) toxicity. In vitro and in vivo islet Tac exposure elicited significant but reversible reduction in insulin secretion in both mouse and human islets. Supplementation with AAGP resulted in improvement of islet survival (Tac\textsuperscript{+} vs. Tac+AAGP, 31.5\% vs. 67.6\%, p<0.01) coupled with better insulin secretion (AUC: Tac\textsuperscript{+} vs. Tac+AAGP, 7.3 vs. 129.2 mmol/L/60 min, p<0.001). The addition of AAGP reduced oxidative stress, enhanced insulin exocytosis, improved apoptosis and also improved engraftment in mice by decreasing expression of IL-1β, IL-6, keratinocyte chemokine and TNF-α. Finally, transplant efficacy was superior in the Tac+AAGP group, and was similar to islets not exposed to tacrolimus, despite receiving continuous treatment for a limited time. Thus, supplementation with AAGP during culture improves islet potency and attenuates long-term tacrolimus induced graft dysfunction.
INTRODUCTION

Islet transplantation outcomes have improved significantly in highly specialized centers, with 5-year insulin-independence rate exceeding 50% (1). However, a proportion of patients require reintroduction of insulin at delayed time-points (2). Numerous factors contribute to limited durability of glycemic control both acutely and chronically. Exposure to diabetogenic immunosuppressive agents is associated with islet functional impairment and graft loss, especially linked to corticosteroid exposure or use of calcineurin-inhibitors (CNI), including tacrolimus (Tac) (3; 4). Tac is used as the most potent mainstay immunosuppressant in most clinical protocols, although diabetogenicity is well documented, accounting for increased incidence of post-transplant diabetes mellitus (PTDM) (5-8). Early peak tacrolimus levels in portal blood after islet transplantation after oral administration may further increase islet-exposure thereby magnifying toxicity (9; 10). Tacrolimus has become ‘a necessary evil’ to prevent rejection and autoimmune recurrence after islet transplantation, but may in part be responsible for limited islet durability, and need for multiple donors for each recipient (11).

Anti-freeze proteins (AFPs) have generated considerable interest for their ability to protect cells under a variety of conditions. They naturally occur in Arctic and Antarctic fish as well as other cold-climate dwelling invertebrates, and are responsible for maintaining cell and tissue function at sub-zero temperatures (12; 13). AFPs were successfully isolated in the 1950s and have demonstrated an ability to non-colligatively lower the freezing temperature of body fluids by binding to ice crystals (12; 14). Early experiments with AFP in the field of organ and tissue transplantation showed promising
results, making them attractive therapeutic candidates to protect cells against harmful conditions associated with the process of procurement-preservation-reperfusion (14). Moreover, benefits have also been demonstrated during cryopreservation of different cells, including islets of Langerhans, with significant improvements in their viability and function when supplemented with AFP during cryostasis (15; 16).

Anti-Aging Glycopeptide (AAGP) is a 580 dalton synthetic AFP analog initially developed by Dr. Geraldine Castelot-Deliencourt-Godefroy (Rouen, France) and later manufactured by ProtoKinetix Inc. (Vancouver, Canada). This new compound has improved stability, is water soluble and has proven to be more potent in terms of cytoprotective capabilities under extreme conditions (pH variations, sudden temperature changes, nutrient deprivation, oxidative stress, UV radiation and inflammation) (17).

In light of this evidence, significant attention is now being directed towards AFP and their potential use in reparative and regenerative medicine, particularly in the field of transplantation. We herein evaluate the cytoprotective capacity of AAGP to protect against the diabetogenic effect of tacrolimus resulting in improved islet engraftment.
RESEARCH DESIGN AND METHODS

Human islet isolation, purification and culture

Human islet preparations were isolated from consenting multi-organ deceased donors, as previously described (18) with intent for clinical transplantation, and were only made available for research when the islet yield fell below that of the minimal mass required. Permission for these studies was granted by the Health Research Ethics Board of the University of Alberta, Edmonton, Alberta, Canada, and after written permission was obtained from donor families.

In vitro evaluation of AAGP

Islet recovery, viability and secretory function

Experiments with human islets in vitro included four groups: a. Control (islets cultured in medium alone) (Tac\(^{-}\)); b. Islets cultured in medium containing AAGP (AAGP); c. Islets cultured in medium containing tacrolimus (Prograf, Astellas Pharma Canada Inc., Markham, ON, Canada) (Tac\(^{+}\)), and; d. Islets cultured in medium supplemented with AAGP (ProtoKinetix) and tacrolimus (Tac+AAGP). Islets were cultured for 24 hours with ± 3mg/mL AAGP before addition of tacrolimus at clinically relevant concentration of 10ng/mL. All four groups were then cultured for an additional 24 hours. Islets were assessed for recovery, viability, insulin release, oxidative stress and cell death. Recovery rate was calculated as the percentage of surviving islets after culture in comparison to the initial count for each condition. Viability was assessed using a fluorescent membrane integrity assay with counter-stains using SYTO® 13 Green and Ethidium bromide (Life Technologies, Burlington, ON, and Sigma-Aldrich, ON) (19-22).
Hormonal islet secretory function was assessed by both static glucose-stimulated insulin secretion (s-GSIS), sequentially performed at low (2.8 mmol/L) and high (16.7 mmol/L) glucose concentrations, and with dynamic islet perifusion (d-IP), as described by Cabrera et al (23). D-IP was performed at 16 min intervals using low (2.8 mmol/L), high x 2 (28 mmol/L), followed by low glucose concentration. Glucose was infused at 100µL/min and results are expressed as fold-change of insulin secretion compared to the low glucose stimulation baseline, normalized for 100 IEQ. For s-GSIS, insulin concentrations in supernatants were measured by ELISA (Mercodia, Uppsala, Sweden). A stimulation index was calculated as the ratio of stimulated to basal insulin secretion normalized by DNA. These insulin secretion studies were always performed in vitro, on cultured human islets.

Apoptosis was assayed by determining quantity of cleaved caspase-3 in the frozen lysates from fixed islets cultured under the above mentioned conditions using a spectrophotometric assay (EMD Millipore. Billerica, MA, USA). Results are expressed as fold-change increase compared to controls.

Islet apoptosis was also examined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (DeadEnd Apoptosis Detection System, Promega, Madison, WI), following formalin fixation, processing and paraffin-embedded. Co-staining with insulin (1:200 concentration of anti-insulin antibody (Dako, Mississauga, ON) and DAPI (Invitrogen Molecular Probes. Eugene, Oregon) was performed to identify graft and nuclei present, respectively. Islet apoptosis was quantified by percentage of positive TUNEL stained area using ImageJ software (freeware ImageJ v1.33 and Cell Counter plug-in, [http://rsb.info.nih.gov/ij]).
**Reactive oxygen species analysis**

Frozen samples from the study groups were assayed for reactive oxygen species (ROS) released into the culture media, using the Acridan Luminogen PS-3 assay (Amershan ECL Plus kit, Fisher Scientific Inc. Ottawa, ON, Canada) (24). Acridan Luminogen PS-3 is excited by reactive oxygen and nitrogen species in the presence of hydrogen peroxide, producing chemiluminescence at 430nm. Media samples were flash-frozen in liquid nitrogen and stored until analyzed. CMRL culture medium alone served as a control, and results were expressed as fold-change increase compared to control.

**Mixed Lymphocyte Reaction (MLR)**

To rule out direct drug inhibition of AAGP and tacrolimus a one-way MLR assay was performed to assess proliferative response of responder T-cells against antigens present on allogeneic stimulator cells. T-cells were isolated from gamma-irradiated splenocytes of BALB/c mice (stimulators) and from C57BL/6 mice (responders). Proliferation was measured by loss of fluorescence intensity using fluorochrome 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen. Eugene, OR), which spontaneously binds to intracellular proteins shared between daughter cells. Controls (Tac\(^-\), AAGP\(^+\)), Tac\(^+\), and Tac\(^+\)AAGP groups were studied. T cell proliferation was measured by flow cytometry after characterizing different subpopulations by cell surface antibody staining with anti-mouse TCR-beta eFluor450, CD4 APC and CD8a APC-eFluor780 (eBioscience, add location). Acquisition was performed on a BD LSR II flow cytometer (BD Biosciences, add location), followed by analysis with FCS Express Data software (De Novo Software, Los Angeles, CA).
Calcium imaging

Measurements of intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) for individual human islets from the different treatment groups were carried out by previously described methods (25; 26) at glucose concentrations of 2.5mmol/L and 25mmol/L. Glucose-stimulated increase in [Ca\(^{2+}\)]\(_i\) was expressed as area under the curve.

Capacitance studies

Measurement of membrane capacitance was performed on islets according to our previously established method (27; 28) to determine the effect of Tac and AGPP on β-cell exocytotic responses. Cells were stimulated with a series of ten depolarizations to activate voltage-dependent Ca\(^{2+}\)-channels. Whole-cell capacitance responses were normalized to initial cell size and expressed as femtofarad per picofarad (fF/pF).

In vivo evaluation

All mice were housed under conventional conditions with access to food and water ad libitum. The care for mice was in accordance with guidelines approved by the Canadian Council on Animal Care.

Transplants with human islets and inflammation analysis

Diabetes was induced chemically on 8-12 week immunodeficient B6.129S7-Rag\(^{1\text{tm1Mom}}\) recipient mice (Jackson Laboratory, Bar Harbor, ME, USA) by intraperitoneal injection of 180mg/kg streptozotocin (STZ, Sigma-Aldrich, ON, Canada). Mice were considered diabetic after two consecutive blood glucose measurements ≥11.3mmol/L (350 mg/dL). Recipients (n=10 per group) received approximately 1,000 IEQ human islets from 3 different isolations. Islets from each isolation were randomly allocated to each group.
(Tac⁻, AAGP, Tac⁺, Tac+AAGP), to control for potential differences in each islet preparation. Islets were transplanted under the kidney capsule as described previously (29). A minimal islet dose was utilized to stress the model and maximize covert toxicity (30-32).

Three mice per group underwent acute graft explantation and were euthanized on day 1 and 7 post-transplant to determine proinflammatory cytokine concentrations (at both time points), cleaved caspase-3 and TUNEL (24h) within the graft. For cytokine and cleaved caspase-3 quantification the islet grafts were excised from the kidney, bisected, with one section flash frozen in liquid nitrogen and stored at -80°C, while the other was formalin fixed and processed for TUNEL quantification. Tissue samples were subsequently lysed in acid buffer as reported previously (30). Cytokine and cleaved caspase-3 determination was adjusted per gram of tissue.

**Pro-inflammatory cytokines and chemokines**

Relevant cytokines and chemokines (IFN-γ, IL-1β, IL-6, IL-10, IL-12, Keratinocyte-derived Chemokine (KC), and TNF-α) were measured using a Multi-Spot Mouse ProInflammatory 7-Plex Ultra-Sensitive kit and analyzed on a SECTOR Imager (Meso Scale Discovery®, Gaithersburg, MD, USA). Results are expressed as absolute values (pg/mL), and normal renal tissue lysate samples from a mouse receiving a sham operation are used as control.

**Apoptosis**

Apoptosis was determined in the excised grafts 24h post-transplant by quantifying cleaved caspase-3 and analyzing percentage of death cells (TUNEL) within the graft.
Caspase-3 concentration was expressed as fold-change increase compared to normal renal tissue lysate samples from a naive mouse.

**Long-term human islet graft function after transplantation in immunodeficient mice**

Non-fasting blood glucose was monitored in the remaining animals three times a week using a portable glucometer (OneTouch Ultra 2, LifeScan, Canada) over 60 days. Normoglycemia was defined as two consecutive readings <11.3mmol/L.

Intraperitoneal glucose-tolerance tests (IPGTT) were conducted 60 days post-transplant to evaluate the capacity of islets to respond to a glucose bolus (3g/kg) after overnight fast. Blood glucose levels were monitored at baseline time 0, 15, 30, 60, 90 and 120 minutes post-injection. All results were compared to blood glucose profiles of naive control non-diabetic mice.

Recovery islet-bearing nephrectomies were performed on day 65 to demonstrate graft-dependent euglycemia. Both cultured islets and recovered grafts were stored at -80°C and processed to measure intracellular insulin content by acid-ethanol homogenization and ultrasonic lysis. The extract was neutralized and insulin measured with ELISA (30).

**Transplants with mouse islets under continuous treatment with tacrolimus**

**Mouse islet isolation**

Pancreatic islets were isolated from 8 to 12 week male BALB/c mice (Jackson Laboratories, Canada) as reported previously (18). Islets were counted and divided in three groups (Tac⁻, Tac⁺ and Tac+AAGP). All islets were incubated for 1h in conditions described above and Tac+AAGP islets were additionally supplemented with AAGP at 3mg/mL during the incubation period.
Recipient syngeneic BALB/c mice were also rendered diabetic with STZ and transplanted after incubation with 500 IEQ ±10% of 90% purity, under the renal capsule (18).

Subcutaneous Micro-Osmotic Pumps (Model 1002, Alzet, Cupertino, CA) were implanted in all mice at the time of islet transplantation to provide continuous delivery of tacrolimus. A first group (short duration) received a pump delivering Tac for 7 days, at a dose of 1mg/kg/day to the relevant groups (Tac⁺, n=6 and Tac+AAGP, n=8), and a second group (long duration) received pumps delivering the CNI for 28 days (Tac⁺, n=6 and Tac+AAGP, n=6). Tac⁻ group (n=10) received pumps loaded with normal saline as placebo. Steady-state Tac levels were monitored selectively from the dorsal tail vein at day 5, and ranged from 10-20ng/ml (clinically relevant range, tandem liquid chromatography-mass spectroscopy, for continuously administered drug) (33).

Animals underwent IPGTT on day 7 (short duration), day 14 (long duration) during treatment course, and again, on day 30 and 40, respectively after CNI treatment cessation. Transplant islet-bearing nephrectomies were performed after tolerance tests to prove graft-dependent function.

**Statistical analysis**

Data are represented as means ± standard error of the mean (SEM). Area under the curve was calculated for GSIS and D-IP, calcium imaging, capacitance measurements and IPGTT, and differences between groups were analyzed with one-way ANOVA with Tukey’s post-hoc test. A p-value <0.05 was considered significant and all the analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).
Results

_AAGP enhances human islets potency in culture and protects islets against acute exposure to tacrolimus_

Isolated human islets from 6 different preparations were cultured in media supplemented with or without AAGP and tacrolimus, as described above. After 48 hours of culture, all groups were characterized for _in vitro_ survival, viability, function, oxidative stress and apoptosis.

After the study period, cells were counted resulting in a greater number of surviving islets in the AAGP supplemented group (71.1%). Exposure to tacrolimus clearly decreased survival, but islets significantly recovered when simultaneously supplemented with AAGP (Tac\(^+\) vs. Tac+AAGP, 31.5% vs. 67.6%, p<0.01) (Figure 1A). There was no difference in cell viability by membrane integrity stain (Figure 1B).

When comparing in-vitro function by D-IP, insulin release was completely suppressed after tacrolimus exposure (AUC: Tac\(^-\) vs. Tac\(^+\), 131 vs. 7.3 mmol/L/60min, p<0.001). However, islet function was fully restored after supplementation with AAGP and comparable to the other culture conditions (Tac\(^+\) vs. Tac+AAGP, 7.3 vs. 129.2 mmol/L/60min, p<0.001) (Figure 1C and D).

_Acute exposure to tacrolimus decreases insulin secretion but not biosynthesis_

Aliquots of 100 IEQ human islets were collected for each group for s-GSIS assay and intracellular insulin content. The Tac\(^+\) group showed significant impairment of insulin secretion, which was not observed in the Tac+AAGP group (stimulation index 1.4 vs. 10.7, p<0.01) (Figure 1E). However, intracellular insulin content remained stable and
comparable throughout groups, demonstrating no changes to biosynthesis of insulin within beta cells (Figure 1F).

**AAGP reduces oxidative stress but does not inhibit tacrolimus function**

Oxidative stress was observed in all groups, but Tac exposure resulted in substantial increase in ROS, which was ameliorated in the presence of AAGP (Figure 2A, n=6, p<0.05).

To confirm that AAGP did not inhibit Tac suppression of T cell proliferation, MLRs were completed with donor splenocytes. The assay measured T cell proliferative response by CFSE staining. As expected, T cell proliferation was significantly decreased in the presence of Tac compared to IgG controls (n=4, p<0.001). Proliferation of CD8+ and CD4+ positive T cells was also significantly decreased in the presence of Tac alone or in combination with AAGP (n=4, p<0.001 in both cases), with no impediment to MLR suppression in the presence of AAGP (Figure 2B, C and D).

**Tacrolimus effect on islet intracellular calcium responses and exocytosis.**

Various studies were performed on human islets to elucidate a potential mechanism of action for the AAGP by characterizing CNI-related injury and its minimization. Intracellular calcium concentrations were measured to determine possible influence of AAGP on glucose-stimulated calcium influx (34). No significant differences in calcium influx were observed between groups (AUC: Tac- 209.4, Tac+ 221.6, Tac+AAGP 208.7 fF/Treatment/2s. p>0.05), suggesting that the protective effect of AAGP was further downstream in the secretory pathway (Figure 3A).

Complementary membrane capacitance studies were performed under similar conditions as an indirect indicator of insulin exocytosis. There was a decreased cumulative
capacitance response or the Tac\textsuperscript{+} group compared to other groups, and a significantly lower AUC when compared to the Tac+AAGP group (AUC: 2.9 vs. 10.5 fF/pF/treatment, p<0.001, \textbf{Figure 3B}).

\textit{AAGP prevents islet apoptosis resulting from in-vitro exposure to tacrolimus}

Exposure to tacrolimus during culture resulted in increased concentration of intracellular cleaved caspase-3 (fold change Tac- 1.9 vs Tac+ 4.3, p<0.05), which corresponded with increased percentage of apoptotic islets (Tac- 18.9\% vs. Tac+ 48.6\%, p<0.01). Conversely, pre-treatment with AAGP prevented tac-induced cell death, showing reduced levels of caspase-3 (Tac+ 4.3 vs. Tac+AAGP 2.2, p<0.05) and fewer number of apoptotic cells (Tac+ 48.6\% vs. Tac+AAGP 26\%, p<0.05) (\textbf{Figure 4A and B}). Representative slides from TUNEL histology are shown in \textbf{Figure 3C}.

\textit{AAGP ameliorates inflammatory response immediately post-transplant}

Minimal mass (1,000 IEQ) human islet transplants were performed in diabetic immunodeficient mice. Grafts from three animals per group (day 1 and day 7 post-transplant) were homogenized and characterized for proinflammatory cytokines and chemokines.

Acute levels of IL-1\beta were significantly increased in Tac\textsuperscript{+} with respect to those in the sham group (18.9 vs. 163.1 pg/g-tissue, p<0.001). Cytokine concentration was however considerably dampened in the Tac+AAGP group (163.1 vs 44.9, p<0.001), with similar excretion behavior on day 7 (269.5 vs. 121 pg/g-tissue, n=3, p<0.001) (\textbf{Figure 5A and B}). Similarly, IL-6 was significantly increased in the Tac\textsuperscript{+} group (1414 vs. 804.7 pg/g-tissue, n=3, p<0.001) but differences were no longer apparent at later time points (\textbf{Figure 5C - D}).
Among the chemokines measured acutely post-transplant, KC secretion, involved in neutrophil recruitment, was significantly overexpressed in Tac+ and again, significantly reduced in the presence of AAGP (85.4 vs. 32 pg/g-tissue, p<0.001, n=3). By day 7 the cytokine was clearing and differences were no longer evident (Figure 5E and F). Tumor necrosis factor levels on the other hand, were not significantly increased on day 1, but became notably different on day 7 (Tac-, 33.9 pg/g-tissue; Tac+, 76.7 pg/g-tissue and Tac+AAGP, 48.7 pg/g-tissue. P<0.001) (Figure 5G and H).

Intra-graft apoptosis showed an increasing trend in cleaved caspase-3 concentration and TUNEL positive cells in Tac+ group, when compared to the rest of the groups, suggesting increased in-vivo cell death after CNI treatment and subsequent AAGP protective effect. However, differences did reach statistical significance (Tac+ vs Tac+AAGP: Fold-change in Caspase-3, 2.9 vs. 1.9, N.S.; TUNEL positive cells: 35.3±26.7% vs 7.9±8%, N.S.) (Data not shown).

**AAGP-supplementation improves human islets transplant function despite tacrolimus exposure**

The remaining transplanted mice (n=7 per group) were followed beyond 60 days. Delayed engraftment was observed as expected in this marginal islet mass model. Blood glucose improved over time in Tac- and Tac+AAGP groups, with the proportion of euglycemic animals being significantly higher when compared to the Tac+ where all mice demonstrated poor function (p<0.05) (Figure 6A and B).

Thirty days post-transplant mice underwent IPGTT to evaluate transplant function. Tac- and Tac+AAGP groups both responded appropriately, but Tac+ remained hyperglycemic at 120 min (AUC: Tac- vs. Tac+AAGP, 92.6 vs. 91.2, p>0.05.; Tac- vs. naïve, 92.6 vs.
71.4, p>0.05.; Tac+AAGP vs. naïve, 91.2 vs. 71.4, p>0.05, and Tac+ vs. Tac+AAGP, 149.8 vs. 91.2, p<0.05, Figure 6C).

All mice reverted to their previous diabetic state following islet-bearing nephrectomy. Insulin content was assessed as a measure of residual islet mass after 30 days. Figure 6D shows significant differences between groups with reduced insulin content in grafts exposed to tacrolimus. Again, presence of AAGP was beneficial in islet protection despite exposure to tacrolimus (Tac+ vs. Tac+AAGP, 30.9 vs. 100.8 ng/mL, p<0.01).

**AAGP-supplementation improves islets transplant function despite continuous recipient treatment with tacrolimus**

In further support of the above findings, syngeneic diabetic mice were implanted with mini-osmotic pumps to model continuous post-transplant Tac treatment to resemble clinical practice. As with the *in vitro* findings, transplanted islets exposed to Tac were unable to effectively secrete insulin nor return mice to euglycemia during the treatment course. The additional presence of AAGP, however, restored normal islet function despite Tac exposure, similar to controls (Figure 7A and B, p<0.001).

Similar findings were observed when utilizing subcutaneous pumps providing Tac treatment for a longer duration (28 days). Again, AAGP-supplemented islets functioned normally and rendered normoglycemia for all animals, despite a single 1h AAGP treatment to islets prior to transplant (Figure 7E and F, p<0.01)

Results were corroborated by IPGTTs performed in both treatment modalities (short and long duration). Tolerance tests performed under Tac treatment showed impaired glucose
control in Tac+ group, whereas Tac+AAGP behaved similar to controls (Figure 7C and G, p<0.001).

Tacrolimus treatment cessation resulted in normalization of graft function and euglycemia in all animals. Repeat IPGTTs at this stage (30 and 45 days, respectively) showed no residual differences between groups (Figure 7D and H).
Discussion

We demonstrate herein that addition of a potent anti-freeze protein, AAGP only to the islet culture media for a 48 hour exposure affords considerable protection of human islet survival and in vitro function. This protective effect is especially pronounced when used to prevent Tac-induced islet toxicity.

Tacrolimus is currently regarded as a mainstay, potent immunosuppressant given to prevent both auto- and alloimmunity after clinical islet transplantation (35). Prolonged exposure to CNI-class immunosuppressants is strongly associated with nephrotoxicity and PTDM in all organ transplants (4).

Tacrolimus is known to impair insulin secretion in the native pancreas, after pancreas and especially after islet transplantation, and is characterized by impairment of early secretion and by decreased biosynthesis. Several associated mechanisms have been defined, including calcineurin/nuclear factor of activated T-cells signaling inhibition (36), insulin gene suppression (37), mitochondrial arrest (38) and decreased post-transplant vascularization (39). In our experimental model, the addition of Tac resulted in striking inhibition of insulin secretion and cell death in vitro, and impaired islet engraftment and function in vivo.

Furthermore, we confirmed islet function impairment after Tac exposure in vitro and we found that AAGP was able to reestablish insulin release despite acute exposure to high-dose Tac.

Increased loss of islets during culture associated with apoptosis was observed after in vitro exposure to tacrolimus. Increase in cleaved caspase-3 and TUNEL staining
indicated significantly higher cell death in Tac⁺ group, however this was diminished after AAGP supplementation.

Islets are highly susceptible to hypoxia throughout all stages of cell procurement, preparation and intraportal transplantation, and relates to their intrinsic oxygen demand and size, especially related to islet seeding density in culture (40). Islets are prone to oxidative stress due to decreased antioxidant capacity (41). These elements contribute to islet loss during culture and post-transplant. Our findings confirm increase in oxidative stress after Tac exposure, with increased extracellular ROS. AAGP supplementation reduced oxidative stress in this model. Similar redox modulation findings have also been noted when using AAGP with other cell lines (17).

*In vivo* studies complemented all *in vitro* findings, which clearly demonstrated that AAGP supplementation suppressed early inflammation and improved islet engraftment with long-term efficacy. AAGP-supplemented islets showed significantly reduced expression of IL-1β and IL-6, along with decreased secretion of KC and TNF, despite exposure to Tac in culture. These cytokines and chemokines are key participants in the post-transplant inflammatory response and subsequent adaptive immunity activation (42), and a vital element in the early clinical post-transplant phase (30). These findings are consistent with previous experiments showing reduced expression of cyclooxygenase-2 expression in HeLa cells exposed to increasing concentrations of IL-1β, in the presence of AAGP (17).

Tac exposure was provided by continuous mini-osmotic pump for 7 and 28 days in syngeneic mice. We chose this approach in selected experiments, as twice-daily oral gavage of Tac would have been too stressful, but wished to maintain sustained clinically
relevant drug exposure for transplanted islets. We observed marked impairment of transplanted islets occurred immediately following Tac exposure, which lasted throughout Tac exposure, but was reversible after withdrawal of Tac. Islets treated with AAGP however, were protected from Tac toxicity, and functioned similar to controls both, in short and long duration treatment groups. Potentially, since a marked and prolonged post-transplant engraftment and functional benefit was observed consistently when AAGP treatment was confined only to the in vitro culture period, this treatment could be readily applied in clinical studies to enhance islet engraftment and function in patients receiving tacrolimus immunosuppression.

In exploring potential mechanisms of action of AAGP, we found no beneficial effect upon insulin synthesis or storage. Furthermore, we did not find an interactive impact of AAGP upon the immunosuppressive properties of Tac. We found that neither Tac nor AAGP affected glucose-stimulated calcium-influx in islets, which is a key element in the insulin secretion mechanism of beta cells. This information is supportive of recently published evidence pointing to a potential tacrolimus mechanistic site further downstream in the secretory pathway (43). Conversely, islet capacitance measurements in the current studies revealed significant differences between Tac+ and TAC+AAGP, findings suggestive of impaired insulin exocytosis in the presence of Tac, which was reversed by AAGP.

In conclusion, supplementation of islets with AAGP during culture enhanced both the quality and yield of post-culture human islets, which translated into improved engraftment, despite the presence of Tac. AAGP also protected islets continuously exposed to Tac post-transplant, with improved efficacy and decreased inflammatory
response. Clinical translation of these findings could potentially offer a means to protect islets both in vitro, and in vivo from diabetogenic immunosuppression after transplantation, as a means to enhance single donor islet engraftment and durable long term function.
Author Contribution

BGL designed and performed experiments, researched data and wrote the manuscript. ARP researched data, performed experiments and contributed to the discussion and reviewed/edited the manuscript. RLP isolated mouse islets and reviewed the manuscript. DO performed D-IP and reviewed manuscript. TK isolated human islets and reviewed manuscript. ABruni, NA and MB researched data. ABautista performed capacitance studies. JMF performed calcium imaging and reviewed manuscript. LGY contributed to study design, provided AAGP and reviewed manuscript. PEM researched data and reviewed manuscript. AMJS (senior author) designed experiments, researched data and reviewed/edited manuscript.

Acknowledgments

BGL is supported through the Izaak Walton Killam Memorial Scholarship, an Alberta Innovated Healthcare Solutions (AIHS) Clinician Fellowship, and from the Canadian National Transplant Research Program (CNRTP). Other funding sources include: the Alberta Diabetes Research Institute Foundation of Canada (DRIFCan), the AIHS Collaborative Research & Innovation Opportunities (CRIQ) Team Award, and from the Alberta Diabetes Institute (ADI). PEM is supported by a Canada Research Chair in Islet Biology. AMJS is supported through a Canada Research Chair in Transplantation Surgery and Regenerative Medicine, and through AIHS as a Senior Scholar.

AMJS is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Disclosure

One author of this manuscript has conflicts to disclose as described by the Diabetes Journal. LGY owns shares in ProtoKinetix, the company that owns the patent rights to the AAGP molecule. The other authors have no conflicts of interest.
Figure Legends

**Figure 1. AAGP improves human islet potency in culture and protects against acute exposure to tacrolimus.** *In vitro* assessment of human islets in culture with or without AAGP supplementation showed (A) significantly higher islet recovery rate after culture in the presence of AAGP. (B) No significant changes in cell viability were found after study period. (C, D) Perifusion curves comparing glucose-stimulated insulin secretion (GSIS) after stimulation with variable glucose concentrations (low=2.8 and high=28 mM), showing severely impaired islet function for the Tac⁻ group and significantly better response for groups treated with AAGP, also seen in the corresponding area under the curve (n=6).

_Tacrolimus impairs insulin secretion without affecting insulin biosynthesis._

GSIS static assay and intracellular insulin content were simultaneously measured on human islets kept in culture. (E) Stimulation index (SI) for group Tac⁻ is significantly decreased in comparison with controls. However, a significant improvement was observed in insulin secretion of Tac+AAGP islets (p<0.01), while no changes were seen in the intracellular content of insulin across the different groups (F). Data points represent mean ± SEM, triplicates from four different preparations. *P<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

**Figure 2. Islets treated with AAGP have decreased oxidative stress.** (A) Human islets in culture had increased concentration of reactive oxygen species when treated with Tac. However, supplementation with AAGP significantly decreases this effect (p<0.05). Oxidative stress was measured by fold-increase in extracellular ROS analyzed with the
Acridan Luminogen PS-3 assay (n=5).

*AAGP effect is not the result of direct drug inhibition with tacrolimus.*

**(B, C and D)** Allogeneic mixed lymphocyte reaction (MLR) was used to evaluate direct drug inhibition. Results show a significant decrease of T cell proliferation in the presence of tacrolimus, AAGP and the combination of both. Hence, no direct inhibition of tacrolimus by AAGP. Data points represent mean ± SEM, n=6, ***p<0.001 and ****p<0.0001.

**Figure 3. AAGP improves insulin release by increasing islet exocytosis.** Human islets were cultured and treated accordingly with and without Tac/AAGP. Insulin secretion impairment for Tac⁺ group was also met by a lower concentration of intracellular calcium **(A).** Differences among groups did not reach statistical significance but further studies showed that normalized membrane capacitance measurements were significantly worse for Tac⁺ islets (blue), indicating impaired exocytosis, whereas measurements were superior and comparable in the Tac⁻ (red) and Tac+AAGP groups (black) (p<0.01 and p<0.001). Data points represent mean ± SEM, triplicates from two isolations.

**Figure 4. AAGP decreases islet loss in culture due to apoptosis, even in the presence of tacrolimus.** Human islets were cultured and treated with Tac/AAGP, accordingly. Cell death due to apoptosis was significantly higher in Tac⁺ by quantification of intracellular cleaved caspase-3 **(A)** and by analysis of TUNEL staining **(B).** Supplementation of media with AAGP was able to restore viability and significantly decrease cell death (p<0.05). **(C)** Representative TUNEL slides of fixed islets from different study groups. Green arrow points to TUNEL positive nuclei.
Figure 5. **AAGP ameliorates inflammatory response immediately post-transplant.** Proinflammatory cytokines and chemokines locally expressed (1 and 7 days after transplantation. (A) Concentrations of IL-1β, IL-6, Keratinocyte chemokine (KC/GRO) and Tumor Necrosis Factor (TNF) were significantly lower in the engrafted islets previously treated with AAGP. Cytokines were measured 24h and 7 days after transplantation, locally to the graft by homogenization (normalized per gram of tissue, n=3). Data points represent mean ± SEM adjusted per gram of tissue, n=3, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Figure 6. **AAGP-supplementation improves islets transplant function despite tacrolimus exposure.** Post-transplant graft function in immunodeficient mice receiving minimal human islet mass (1,000 IEQ). Islets were previously treated with or without AAGP and tracrolimus accordingly. Horizontal continuous line at 11 mM indicates the normoglycemia limit. (A) Pooled blood glucose profiles and (B) percent of mice reaching euglycemia, demonstrating long-term graft function (60 days) with a non-functioning graft for the Tac⁺ group. Graft-bearing nephrectomy was performed on day 60 to demonstrate graft-dependent euglycemia. (C) Intraperitoneal glucose tolerance test (IPGTT) to evaluate metabolic response after receiving a glucose bolus. Tac⁺ group mice were intolerant to high glucose corresponding also to less residual insulin content (D) when grafts were removed after 60 days of transplant. Data points represent mean ± SEM adjusted per gram of tissue, (Tac⁻ n=6, Tac⁺ n=3 and Tac+AAGP n=7), *p<0.05, **p<0.01 and ***p<0.001.

Figure 7. **AAGP-supplementation improves islets transplant function despite continuous (short and long duration) tacrolimus treatment.** Post-transplant graft
function in mice receiving syngeneic full mass (500 islets) islet transplant. AAGP was added to the culture media 1 hour prior to transplant and tacrolimus was administered via a subcutaneous osmotic pump (implanted during the same procedure) at a continuous rate of 1mg/kg/day. (A) Pooled blood glucose profiles of animal over 40 days with clear dysfunction for Tac + islets during the presence of Tac. Vertical dotted line indicates tacrolimus treatment cessation at day 7 and marks a gradual recovery of Tac + grafts. Horizontal continuous line at 11 mM indicates the normoglycemia limit. Graft recovery nephrectomy was again performed on day 30. (B) Mean time-to-euglycemia after transplant showing Tac+AAGP mice reversing diabetes earlier (p<0.001, log-rank, Mantel-cox test). Finally, glucose tolerance tests showed a significant difference in graft response for Tac + when mice were receiving tacrolimus (7 days) and when recipient were CNI free. These differences were not observed in the Tac+AAGP group. (C) Glucose tolerance tests of mice receiving continuous treatment with tacrolimus showing impairment for Tac+ group, which is fully reversed once the CNI treatment is ceased (D).

**Figure 7 (E – F)** A similar experiment was conducted with long duration subcutaneous pumps providing tacrolimus during 28 days. Results show a consistent and significant difference in immediate post-transplant function for mice receiving AAGP supplemented islets.
References


levels of sirolimus and tacrolimus in islet transplant recipients: local immunosuppression or islet toxicity? Transplantation 2003;76:1623-1625


**A**

![Graph](image)

**B**

![Graph](image)

**C**

![Images](image)

*Insulin TUNEL+ Nucleus*

*Diabetes*
Diabetes

**A** IL-1β concentration (pg/g tissue) for 24h:

- Sham
- AAGP
- Tac
- Tac+ AAGP

**B** IL-1β concentration (pg/g tissue) for 7 days:

- Sham
- AAGP
- Tac
- Tac+ AAGP

**C** IL-6 Concentration (pg/g tissue) for 24h:

- Sham
- AAGP
- Tac
- Tac+ AAGP

**D** IL-6 Concentration (pg/g tissue) for 7 days:

- Sham
- AAGP
- Tac
- Tac+ AAGP

**E** KC/GRO concentration (pg/g tissue) for 24h:

- Sham
- AAGP
- Tac
- Tac+ AAGP

**F** KC/GRO concentration (pg/g tissue) for 7 days:

- Sham
- AAGP
- Tac
- Tac+ AAGP

**G** TNF-α concentration (pg/g tissue) for 24h:

- Sham
- AAGP
- Tac
- Tac+ AAGP

**H** TNF-α concentration (pg/g tissue) for 7 days:

- Sham
- AAGP
- Tac
- Tac+ AAGP