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Thrombomodulin Improves Early Outcomes After Intraportal Islet Transplantation

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Primary islet nonfunction due to an instant blood mediated inflammatory reaction (IBMIR) leads to an increase in donor islet mass required to achieve euglycemia. In the presence of thrombin, thrombomodulin generates activated protein C (APC), which limits procoagulant and proinflammatory responses. In this study, we postulated that liposomal formulations of thrombomodulin (lipo-TM), due to its propensity for preferential uptake in the liver, would enhance intraportal engraftment of allogeneic islets by inhibiting the IBMIR. Diabetic C57BL/6J mice underwent intraportal transplantation with B10.BR murine islets. In the absence of treatment, conversion to euglycemia was observed among 29% of mice receiving 250 allo-islets. In contrast, a single infusion of lipo-TM led to euglycemia in 83% of recipients ($p = 0.0019$). Fibrin deposition ($p < 0.0001$), neutrophil infiltration ($p < 0.0001$), as well as expression TNF-α and IL-β ($p < 0.03$) were significantly reduced. Significantly, thrombotic responses mediated by human islets in contact with human blood were also reduced by this approach. Lipo-TM improves the engraftment of allogeneic islets through a reduction in local thrombotic and inflammatory processes. As an enzyme-based pharmacotherapeutic, this strategy offers the potential for local generation of APC at the site of islet infusion, during the initial period of elevated thrombin production.

Key words: Engraftment, inflammation, islet transplantation, thrombomodulin, thrombosis

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Introduction

Despite the promise of islet transplantation, primary nonfunction (PNF) and early nonimmune islet destruction, which have been observed both in animal models and in clinical trials, remain major hurdles in islet transplantation. Notably, islets from two to four donor organs are typically required to reverse diabetes in a single patient, placing a significant burden on an already limited donor organ supply (1). Moreover, a requirement for successive islet infusions within the portal bed necessitates reinterventions with increased costs, the attendant risk of periprocedural morbidity, and has been associated with increasing portal vein pressures that may indicate the development of a presinusoidal form of portal hypertension. PNF may be the consequence of poor functional quality of the grafted tissue, an inadequate mass of transplanted islets, or lack of vascularization of the graft. However, substantial evidence now suggests that exposure to an early, nonimmune inflammatory injury is largely responsible for the observed destruction of islets and may well amplify subsequent immune reactions (2–6).

Although activation of the graft microenvironment by endotoxin (7,8) and lipopolysaccharides have been postulated to contribute to induction of a local inflammatory response, Korsgren and colleagues have demonstrated that an acute blood mediated inflammatory reaction is initiated upon intraportal infusion of islets (9,10). Specifically, in animal models and in recent clinical reports, marked activation of coagulation has been noted after islet infusion, despite the presence of heparin in the infusate, as indicated by increases in thrombin-antithrombin (TAT) complexes, prothrombin activation fragments and fibrinopeptide A. Notably, thrombin is a direct mediator of inflammation, acting as a chemoattractant for neutrophils and monocytes and stimulating endothelial cells to express monocyte chemoattractant protein-1 (MCP-1) and other chemokines. Thrombin also induces endothelial cell expression of ICAM-1, VCAM-1, E- and P-selectin, as well as platelet activating factor, all of which leads to further recruitment of platelets and leukocytes to the graft site (11). Likewise, by-products of the thrombin response, including fibrinogen degradation products and fibrin, also act as chemoattractants and serve to localize this inflammatory response by adhesion-dependent processes. Furthermore, thrombin activated endothelial cells and leuokocytes express oxygen free radicals, IL-β, TNF-α, IFN-γ and iNOS,

which can damage islets, inducing either functional impairment or death (12).

Thrombomodulin (TM), a 60 kD type I transmembrane protein that forms a 1:1 molar complex with thrombin, exerts pronounced inhibitory effect on thrombotic, inflammatory and redox related responses initiated in response to thrombin generation (13). TM binds thrombin and switches off all of its known procoagulant/proinflammatory functions, channeling the catalytic power of the enzyme into complex anticoagulant/antiinflammatory activities. Specifically, thrombin bound to TM is no longer capable of cleaving fibrinogen, nor is it able to activate factors V or VIII or platelets. It is particularly noteworthy, however, that TM significantly enhances the rate of thrombin inactivation by ATIII (~8-fold) and dramatically accelerates (~20 000-fold) the ability of thrombin to activate protein C (APC). Significantly, the biological activity of TM appears intimately tied to its presence within a lipid bilayer. For example, the capacity of membrane fractions of TM to generate APC decreases upon solubilization with most detergents.

It is notable that a variety of proinflammatory cytokines downregulate endothelial cell expression of TM with a concomitant decrease in APC production. Indeed, Kume (14), Terada (15) and Mochida (16,17) have all observed that inflammatory stimuli decreases TM expression in hepatic sinusoidal endothelial cells with fibrin deposition and microthrombus formation *in vivo*. Given these observations, we sought to examine liposomal formulations that consist of TM integrated within lipid bilayers. In this report, we postulated that the capacity of TM to limit early, nonimmune inflammatory injury will enhance islet engraftment and thereby reduce requirements for donor islet mass.

To our knowledge, this report is the first to examine the capacity of exogenously administered TM to enhance islet engraftment in a murine model of intraportal islet transplantation. Given that the thromboregulatory activity of TM is enhanced by the presence of membrane lipids, liposomal formulations that consist of TM integrated within lipid bilayers were produced. Significantly, the ability of human islets to induce platelet aggregation and activation, as well as thrombin production was inhibited by TM containing lipid vesicles. In addition, TM vesicles enhanced islet engraftment *in vivo*, which was associated with a marked reduction in intraportal fibrin formation, neutrophil infiltration and cytokine production.

Research Design and Methods

Animals

Male C57BL/6J (B6) and B10.BR-H2k H2-T18a/SgSnJ (B10) mice (8 weeks old, Jackson Laboratory, Bar Harbor, ME) were used as islet recipients and donors, respectively. The B10 to B6 islet allograft model was initially described by Kaufman et al. (4), with the exception that the renal subcapsule was used as the site for islet transplantation. All animal studies followed local guidelines at Emory University. The B6 mice were made chemically diabetic by i.p. injection of 200 mg/kg streptozotocin in citrate buffer saline and screened as previously described for the development of diabetes. Mice whose nonfasting blood glucose was over 250 mg/dL on two consecutive measurements were considered diabetic (18).

Preparation of TM vesicles

Large unilamellar vesicles (LUV) were prepared from a lipid solution of 12 mM 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC; Avanti Polar Lipids, Inc., Alabaster, AL) in PBS (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.4) by four successive freeze/thaw/vortex cycles using liquid N_2 and a 45°C water bath. A total of 20 μg of rabbit TM was added to 100 μL of the lipid solution and mixed gently for 1 h at room temperature before it was extruded 21 times, each through two back-to-back 600 nm and then 100 nm polycarbonate filters (19).

Islet isolation

B10 mouse pancreata were removed after distension with collagenase P (1 mg/mL; Roche, Indianapolis, IN) through common bile duct. Following digestion, islets were purified by a Ficoll discontinuous gradient (1.108, 1.096 and 1.037; Mediatech Inc, Herndon, VA). Isolated islets were cultured for 48 h in RPMI 1640 supplemented by 10% FCS, L-glutamine (2 mM), and penicillin (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL; Mediatech Inc, Herndon, VA). Viability was evaluated using a Live/Dead Cell Viability/Cytotoxicity Kit (Molecular Probes, Inc., Eugene, OR) and only isolations with >90% viability were used for transplantation.

Islet transplantation

Diabetic B6 mice underwent intraportal islet transplantation after receiving ketamine (95 mg/kg) and xylazine (5 mg/kg) IM for anesthesia. In brief, a predetermined number of B10 islets were infused in a total volume of 200 μL into the recipient liver through the portal vein using a 27 Ga insulin syringe, as previously described (20).

Experimental groups

Mice undergoing islet transplantation were monitored by measuring nonfasting blood glucose daily for 2 weeks with using an ACCU-CHECK glucose monitor. Euglycemia was defined as nonfasting blood glucose less than 200 mg/dL on 2 consecutive days. B6 diabetic mice were randomly assigned into two groups that received either islets alone or islets along with TM at 1 mg/kg IV administered 2 min before transplantation. The dose of liposomal formulations of thrombomodulin (lipo-TM) was selected, in part, based on prior studies of this formulation, which demonstrated efficacy in a murine model of tissue factor induced pulmonary thromboembolism.

Histopathologic examination

Livers were recovered 6 h after intraportal transplantation of 500 islets per mouse from both TM treated and nontreated groups, fixed in 10% formalin, and embedded in paraffin. Five-micron step-sections at 100 μm intervals were obtained. Five consecutive sections were collected from each step section for histological examination. Polymorphonuclear neutrophil (PMN) and fibrin staining were performed after deparaffinization and rehydration using standard immunohistochemical procedures. A commercially available kit (91-C, Sigma-Aldrich, St. Louis, MO) was used to stain for neutrophils using the napthol AS-D chloroacetate esterase procedure. Biotinylated goat antimouse fibrinogen antibody (Accurate Chemical & Scientific Corporation, Westbury, NY) was used to stain for fibrin staining after quenching endogenous peroxidase activity using Dual Endogeneous Enzyme Block (Dako, Carpinteria, CA), blocking endogenous avidin and biotin with the Biotin Blocking System (Dako), and limiting nonspecific binding using 10% normal goat serum (Vector Laboratories, Burlingame, CA). At least 50 islets from each liver sample were examined for fibrin and neutrophil accumulation

by observers blinded to the study. Fibrin deposition around islets was semi-quantitatively analyzed using a scoring scheme (0: no fibrin; 1: mild; 2: moderate and 3: severe fibrin deposition). Neutrophil infiltration was characterized by counting PMNs around individual islets.

Real-time RT-PCR

Liver samples (n = 4–5 livers per group) were recovered 24 h after intraportal transplantation of 500 islets and subjected to total RNA extraction to determine levels of TNF-α and IL-1β mRNA. Three to four tissue segments (30-40 mg/segment) were obtained from visibly ischemic regions of each liver and processed using Trizol reagent (Invitrogen, Carlsbad, CA). RNA was digested using RNase-free DNase (Qiagen, Valencia, CA) and extracted using an RNeasy Mini kit (Qiagen). cDNA was synthesized from 1 μg of total RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). TNF-α and IL-1β expression were investigated using appropriate primers and probes (Taqman Universal PCR MasterMix, Taqman Gene Expression Assays, Applied Biosystems), 50 ng of reversed-transcribed total RNA in a total volume of 20 μL. PCR was run in a 7900HT Sequence Detection System (Applied Biosystems). The relative amount of TNF-α and IL-1β mRNA was normalized to the expression level of 18S and to the corresponding mRNA level in normal livers that did not receive islets or TM. The relative fold increase was calculated using the comparative threshold cycle method.

Pharmacokinetic analysis of liposomal formulations of TM

Blood was obtained from the inferior vena cava of B6 mice in a 3.8% standard sodium citrate medium (9 parts blood, 1 part anticoagulant) immediately after euthanasia by CO_2 asphyxiation. Blood was centrifuged at 8000 rpm for 8 min and pooled mouse plasma prepared from 30 mice. The anticoagulant activity of liposomal formulations of TM was measured and an in vitro dose-response calibration curve defined by determining the activated partial thromboplastin time (APTT) using a STart 4 semi-automated hemostasis analyzer (Diagnostica Stago, Parsippany, NJ). For *in vivo* studies, TM vesicles were administered to B6 mice at 1 mg/kg via intravenous infusion through the jugular vein after induction of anesthesia by IM administration of ketamine and xylazine. Blood samples were collected in tubes containing 3.8% sodium citrate medium (9 parts blood, 1 part anticoagulant) prior to and 5, 30, 60, 150 and 300 min after TM vesicle administration. Plasma was prepared, APTT measured, and equivalent plasma concentration of active TM estimated using the TM-APTT calibration curve. The data were fitted to single and double exponential decay models to determine pharmacokinetic parameters (Matlab software, The MathWords, Natick, MA 21).

Tubing loop model of blood-islet interactions

A tubing loop model of human blood- islet interactions was used to examine the effects of TM vesicle formulations (9,10). Human islets (5000 IEQ) were suspended in 100 μL of either TM-containing vesicles or empty vesicles suspended in PBS. Islets were transferred to loops comprised of heparin-bonded PVC tubing (6.3 mm ID, 40 mm length, Corline System AB, Uppsala, SE, Sweden). Fresh human blood was obtained from healthy volunteers via venipuncture, collected into heparin-bonded 60 mL syringes (Corline Systems). A total of 7 mL of blood was transferred to each loop containing human islets at 5000 IEQ and TM at 700 μg/mL, resulting in a final TM concentration of 10 μg/mL in each loop. This dose approximates the blood concentration anticipated after infusion 1 mg/kg of TM in a 30 mg mouse. A loop containing human islets in 100 mL of PBS was run as a negative control. To simulate portal blood flow, loops were rocked at 37°C to generate a flow rate of approximately 45 mL/min. After 1 h, sodium citrate was added to quench reactions and blood samples collected for analysis. Platelet, white blood cell and lymphocyte counts were determined using a Beckman Coulter ACT. Plasma was analyzed to determine levels of thrombin-antithrombin III, β-thromboglobulin and prothrombin fragment 1 + 2 using commercially available ELISA kits.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Comparison between groups was performed by a Student's t-test. Statistical significance was established at $p < 0.05$. Analysis of eugylcemic conversion over time was performed by Kaplan–Meier method with a Logrank test to assess statistical significance (Prism Software, GraphPad, Inc., La Jolla, CA).

Results

Liposomal formulations of TM improve engraftment of islet allografts after intraportal transplantation

In the absence of adjunctive treatment, allotransplantation of 150 B10 islets was insufficient to correct streptozotocin-induced diabetes (0/12). Euglycemic conversion was achieved in 29% (4/14) of recipients transplanted with 250 islets in the absence of TM liposome infusion; typically between 3 and 7 days after transplantation (mean ± SD: 4.5 ± 0.96 days; Figure 1A–D). In contrast, treatment with TM increased the proportion of mice that attained euglycemia to 83% (10/12), with normalization of blood sugars occurring as early as 1 day after transplantation and as late as six (mean ± SD: 3.3 ± 1.5 days). The median time for 50% of the population receiving TM liposomes to become euglycemic was 4 days. Logrank analysis of the two populations confirmed a significant benefit from treatment with TM (chi-square 9.678, $p = 0.0019$). Moreover, a trend toward increased allograft survival was noted among mice receiving TM. Specifically, long-term (>100 days) eugylcemia was achieved in 42% (5/12) of mice receiving TM as compared to only 7% (1/14) of graft recipients that did not receive TM ($p = 0.06$). Similarly, duration of islet graft function was 72 ± 29 days in the presence of TM, but 34 ± 41 days in the absence of treatment.

TM reduces fibrin deposition and neutrophil infiltration after intraportal islet transplantation

Livers were recovered and examined for both fibrin and neutrophil infiltration in the vicinity of intraportal islets 6 h after transplantation of 500 islets per mouse. Using a semi-quantitative scoring scheme (Figure 2), we observed that treatment with TM reduced peri-islet fibrin deposition from an average score of 2.0 ± 0.2 to 1.0 ± 0.2 ($p < 0.0001$; Figure 3). Indeed, 40% of islets in the TM-treated group had a fibrin score of 0 compared to only 10% in the no treatment group. Likewise, neutrophil infiltration was reduced from 49.4 ± 4.4 PMNs/islet to 19.3 ± 5.7 PMNs/islet among animals receiving an infusion of liposomal TM ($p < 0.0001$; Figure 4).

Real-time RT-PCR

Administration of TM significantly decreased the expression of proinflammatory cytokines, TNF-α and IL-1β, as revealed by an analysis of mRNA in recipient livers 24 h after transplantation (Figure 5). Transplantation of islets, without TM administration, resulted in a fold increase of 5.8 ± 1.4 and 6.7 ± 1.5 in TNF-α and IL-1β mRNA expression, respectively. In contrast, infusion of TM significantly reduced the expression of TNF-α and IL-1β mRNA to 1.5 ± 0.3 and

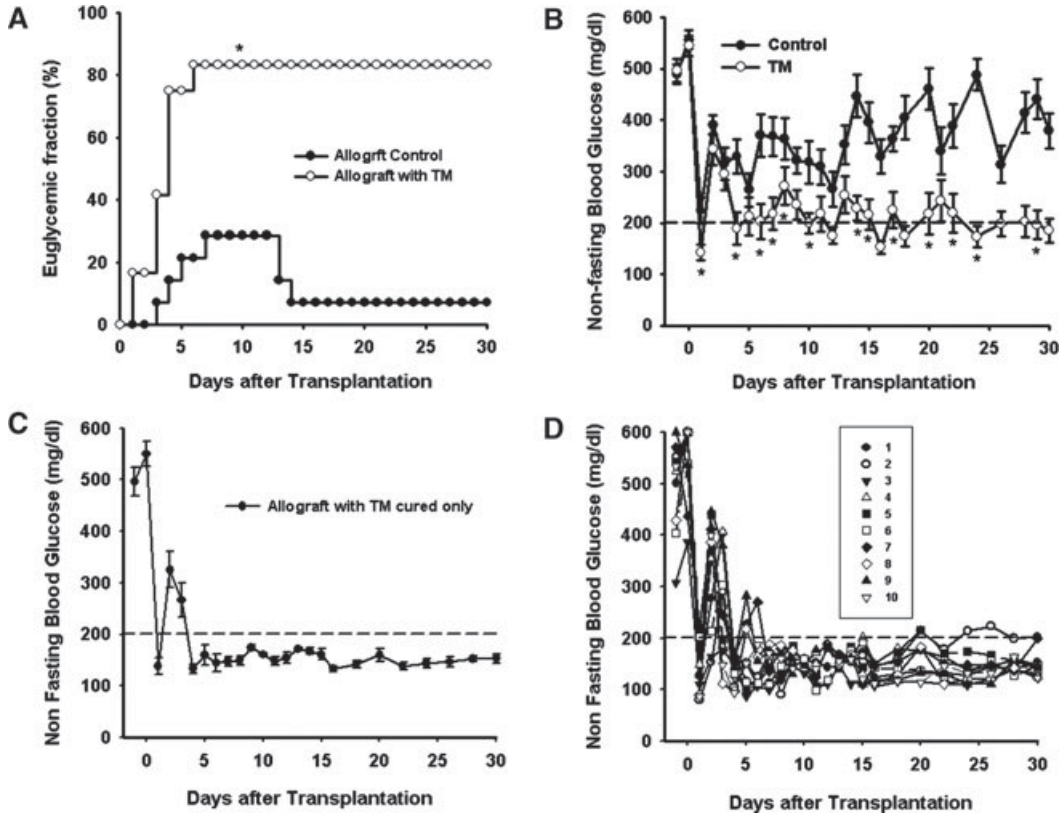

Figure 1: TM improved the engraftment of islet allografts after intraportal transplantation. (A) Pancreatic islets from B10 mice were transplanted into male B6 mice that were rendered diabetic by streptozotocin (200 mg/kg IP). Diabetic animals received either (i) 250 islets alone (n = 14) or (ii) 250 islets along with TM/lipid vesicles (n = 12) by intraportal injection. In the group receiving TM/lipid vesicles, TM at a dose of 1 mg/kg was administered IV via the jugular vein 2 min prior to islet infusion. Serial blood glucose levels were measured and conversion to euglycemia was defined as glucose levels <200 mg/dL for >2 consecutive days (*$p = 0.0019$). (B) Nonfasting blood glucose levels (mean ± standard error) are plotted as a function of time after transplantation for all mice enrolled within each group (*$p < 0.05$ by Student's t-test for 250 islets vs. 250 islets + TM). (C) Nonfasting blood glucose levels (mean ± standard error) are plotted as a function of time for mice receiving TM/lipid vesicles that have undergone a successful transplant (nonfasting blood glucose <200 mg/dL on 2 consecutive days). (D) Nonfasting blood glucose levels are plotted as a function of time for individual mice receiving TM/lipid vesicles that have undergone a successful transplant (nonfasting blood glucose <200 mg/dL on 2 consecutive days).

1.7 ± 0.2, respectively, similar to levels observed in normal livers. Statistical analysis confirmed that the reduction in both TNF-α and IL-1β mRNA expression was statistically significant ($p < 0.03$).

Pharmacokinetic analysis of TM

Liposomal TM was added at varying concentrations to pooled mouse plasma and APTT measured to establish a dose-response curve (Figure 6A). In this manner, *in vivo*

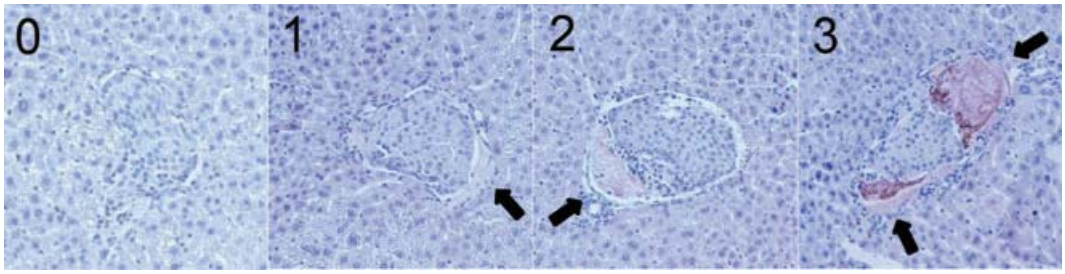

Figure 2: Fibrin deposition after islet transplantation. Scoring system (0: no fibrin; 1: mild; 2: moderate and 3: severe fibrin deposition) used to define the magnitude of peri-islet fibrin deposition after intraportal transplantation. The black arrow indicates fibrin depositions around the islet.

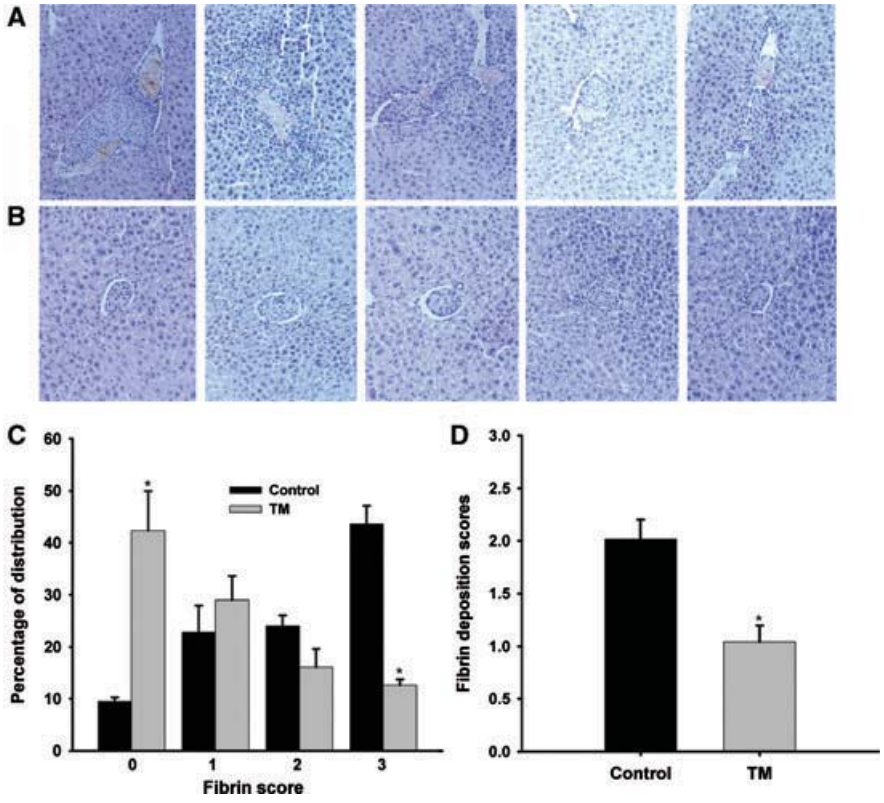

Figure 3: Fibrin deposition after intraportal transplantation of allogeneic mouse islets. Images of islets infused intraportally in the absence (A) or presence (B) of TM/lipid vesicles, 20× magnification. (C) Distribution of fibrin scores 6 h after intraportal transplantation of allogeneic mouse islets (500 islets/mouse) with or without TM ($n = 5$). (D) Fibrin scores for each group expressed as mean ± SD, *$p < 0.0001$ ($n = 5$).

levels of APTT could be used to define an *effective* concentration of active TM after infusion and related pharmacokinetic parameters calculated (Figure 6B–D). After infusion of liposomal TM, changes in APTT provided an estimate of effective plasma levels of active TM over time. The pharmacokinetic half-life estimated from measurements of APTT fit to a single exponential model demonstrated a mean half-life of 86.6 min ($R = 0.94$). Using a biexponential model, fitted curves revealed a mean half-life for the distribution phase ($t_{1/2\alpha}$) of 1.3 min and the elimination phase ($t_{1/2\beta}$) of 70.5 min ($R = 0.99$). These data demonstrate that the systemic biological activity of a liposomal formulation of TM has dissipated 6–7 h after an initial bolus infusion.

Loop model with human islets

A tubing loop model was used to investigate the effect of TM on thrombotic reactions mediated by human islets in contact with fresh, nonanticoagulated whole human blood. Despite the presence of empty lipid vesicles, human islets initiated a significant thrombotic response characterized by thrombin generation and platelet activation, which was substantially inhibited by addition of lipo-TM (Table 1). The presence of islets resulted in a ~500 fold increase in thrombin-antithrombin III (TAT) production relative to control loops without islets. TM reduced TAT levels by 98%, though TAT levels remained statistically higher than control loops ($p < 0.01$). Similarly, levels of prothrombin fragment 1 + 2 were elevated nearly 200 fold in the presence of islets, an effect, which TM reduced by 95% and rendered statistically similar to control loops ($p > 0.01$). Additionally, islet-blood contact induced significant platelet activation, as evidenced by a significant increase ($p < 0.01$) in the release of β-thromboglobulin (β-TG) and platelet consumption. TM reduced β-TG levels approximately threefold and significantly increased platelet count ($p < 0.01$). Moreover, lymphocyte and white cell counts were also reduced

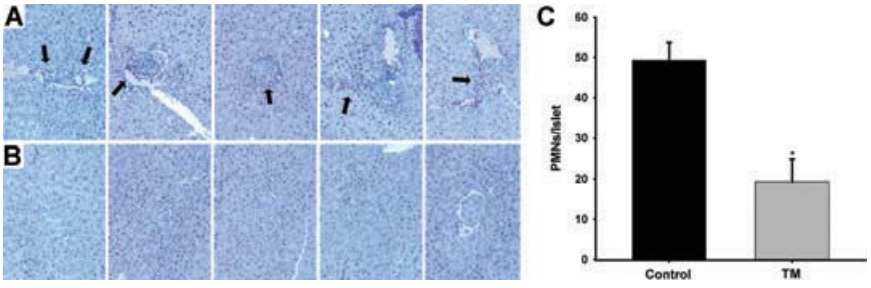

Figure 4: Neutrophil infiltration of allogeneic islets after intraportal transplantation. Images of islets infused intraportally in the absence (A) or presence (B) of TM/lipid vesicles, 20× magnification. Black arrows indicate neutrophil accumulation identified by Napthol AS-D chloroacetate esterase staining. (C) Neutrophils/islet for each group are expressed as mean ± SD, *$p < 0.0001$ ($n = 5$).

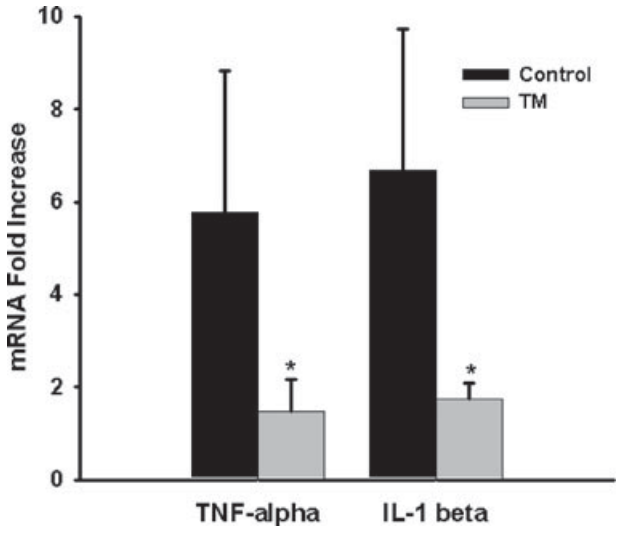

Figure 5: Thrombomodulin suppressed local inflammatory responses after intraportal islet transplantation. Five hundred B10 mouse islets were transplanted with or without TM/vesicles. Livers were recovered 24 h after transplantation. mRNA expression of TNF-α and IL-β was quantified by real-time RT-PCR. Data are presented as mean ± SD, *$p < 0.05$ ($n = 4–5$).

due to entrainment of cells in large thrombi that formed in loops containing islets. TM inhibited thrombus formation, thereby, limiting changes in lymphocyte and white blood cell counts.

Discussion

Marked activation of coagulation has been noted after islet infusion, despite the presence of heparin in the infusate, which leads to significant levels of early islet destruction, as well as, overt and subclinical episodes of portal vein thrombosis (22,23). Prothrombotic triggers include the expression of tissue factor either by transplanted islets or by locally injured endothelial cells (9,10). As a consequence of thrombin generation, activated platelets bind to the islet surface and further amplify the coagulation cascade. While the protein C pathway is a critical physiologic mechanism to limit local thrombin formation, proinflammatory cytokines, such as interleukin-1 (24) and TNF-α (25), downregulate the expression of TM on endothelial cells with a concomitant decrease in APC production. Of particular relevance to intraportal islet transplantation, Kume (14), Terada (15) and Mochida (16,17) have all observed that inflammatory stimuli decreases TM expression in hepatic sinusoidal endothelial cells with fibrin deposition and microthrombus formation *in vivo*.

In recognition of the prothrombotic effects of intraportal islet infusion, most centers performing allogeneic islet transplantation currently use systemic heparin at the time of transplantation. Heparin is usually administered as a bolus dose of ~35 U/kg body weight, corresponding to ~2500 U for a 70-kg person (~0.5 U/mL blood) (26). Korsgren and colleagues (27) have observed that heparin prevented islet-induced coagulation in an *ex vivo* model, but at a eight-fold higher concentration than that used clinically (4 U/mL blood). Furthermore, despite heparin administration at 4 U/mL blood, extensive platelet and fibrin formation, as well infiltration of CD11b+ cells continued to be observed on blood-exposed islets. Finally, even if one were to accept the risk of bleeding complications to be anticipated at a dosing level of 300 U heparin/kg, systemic heparin has a half-life of 1 h and is therefore active for only a few hours. Thus, the potential therapeutic impact of intravenously administered heparin is limited both by its systemic anticoagulant activity that increases the risk of bleeding complications and short half-life.

As an alternative strategy, Contreras et al. (28) have reported that exogenous APC, at a 10-fold higher dose than that recommended for clinical use, reduces the loss of functional islet mass in a murine model of intraportal islet transplantation. APC together with its cofactor protein S inactivates two coagulation factors, Va and VIIIa, thereby preventing the generation of Xa and thrombin. As such, APC inhibits mononuclear phagocyte (MØ) activation and production of proinflammatory cytokines, such as TNF-α and IL-1β, which are known to be cytotoxic to islets (29–32), and limits neutrophil binding to selectins (29–33). APC also suppresses MØ-dependent proliferative responses of T cells, inhibits mixed lymphocyte responses of human and rat mononuclear cells, and prolonged xenograft survival in a guinea pig to rat cardiac transplant model (32). Significantly, primate studies have shown that activation of the protein C anticoagulant pathway carries little bleeding risk (34). When APC was infused at doses sufficient to prevent venous or arterial thrombosis, excessive bleeding from surgical wounds did not occur. Nonetheless, the half-life of APC is approximately 10–20 min, which would necessitate repetitive dosing for a sustained effect (35). As an alternative approach, several studies have documented that various forms of solubilized TM are capable of activating protein C *in vivo*. For example, Kumada et al. (36) observed that TM prolonged the survival of mice in a model of thrombin-induced thromboembolism. Other studies have demonstrated that administration of TM reduces endotoxin induced lung injury (37,38) and thrombosis from disseminated intravascular coagulation (DIC) in rats and monkeys (39–41), limits thrombosis in an arteriovenous shunt model in rats (42), attenuates thrombotic glomerulonephritis in rats (43) and decreases trauma induced spinal cord injury (44). Early phase clinical studies have recently noted that soluble TM may promote the resolution of DIC (45) and reduce the incidence of deep venous thrombosis after hip replacement (46). Prior studies have confirmed that instant blood mediated inflammatory reaction (IBMIR) is a barrier to engraftment in murine models of intraportal islet transplantation (28,47–49). This report is

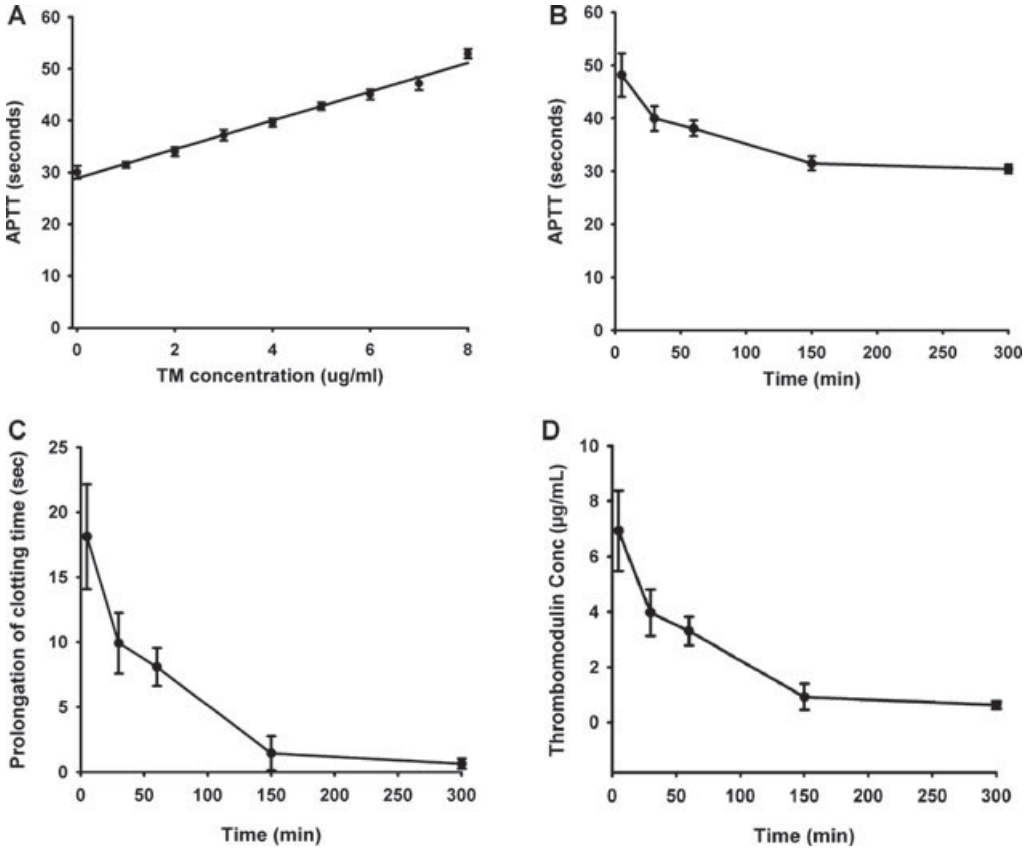

Figure 6: Pharmacokinetic analysis of liposomal formulations of thrombomodulin. (A) In vitro calibration curve of thrombomodulin concentration in B6 mouse plasma (n = 10) vs. APTT (mean ± SD). Pharmacokinetic profile after infusion of liposomal TM in B6 mice as determined by (B) changes in APTT, (C) prolongation of clotting time (PTT – 30.02 ± 1.21; mean ± SD; n = 10) and (D) estimated concentrations of bioactive TM (mean ± SD, n = 4 mice for each time point).

the first to demonstrate that TM enhances engraftment in an allograft model of islet transplantation. Admittedly, TM may also have an effect on alloimmunity. Further investigations to evaluate this possibility are the subject of ongoing studies.

Lipo-TM offer several advantages as compared to direct administration of APC or soluble TM. First, lipid vesicles rapidly localize to sites containing reticuloendothelial cells, including the liver, lung and spleen (50). While uptake by the reticuloendothelial system (RES) is considered a

Table 1: Thrombotic activity of human islets in the presence or absence of TM liposomes

		60 min		
			Islets	
	0 min	Control	TM Vesicles	Empty Vesicles
n	2	4	4	4
Platelets ($\times 10^3/\mu L$)	270 ± 84	230 ± 52	160 ± 45[1]	3.3 ± 0.91[2]
White blood cells ($10^3/\mu L$)	8.5 ± 2.7	8.1 ± 2.5	7.8 ± 2.3	3.6 ± 1.4
Lymphocytes ($10^3/\mu L$)	2.2 ± 0.10	2.5 ± 0.05	2.5 ± 0.06	1.6 ± 0.54
Thrombin-antithrombin III (µg/L)	29 ± 10	67 ± 12	650 ± 140[1]	33 000 ± 6800[2]
β-thromboglobulin (IU/mL)	390 ± 350	1000 ± 100	3200 ± 1300	9300 ± 780[2]
Prothrombin F1 + 2 (pmol/L)	270 ± 95	320 ± 39	3000 ± 1700	59 000 ± 17 000[2]

Data are n, mean ± SD.
Control loops contained blood and PBS loading solution, but no islets.
[1]Significant difference ($p < 0.01$) when compared with the control loop.
[2]Significant difference ($p < 0.01$) when compared with loops containing islets and TM-vesicles

disadvantage for liposome based drug delivery where long circulating times are desired, liposomal localization in the liver provides a mechanism for initially concentrating TM and presumably APC production at the site of maximum thrombin production. Thus, the rapid resolution of TM's systemic effect, as evident by the normalization of APTT within 2 h of infusion, does not preclude the persistence of a local effect within the liver. Indeed, a second advantage of this approach is inherent to an enzyme-based pharmacotherapeutic, which provides a means for prolonged generation of APC at the site of islet infusion, as long as exogenous TM remains active and elevated levels of thrombin are being produced. We note that in our investigations, liposomal TM was administered via the jugular vein, which may have resulted in a substantial first pass effect due to uptake in the lung. It is possible that a greater effect could have been achieved if infused prior to or along with the islets directly into the portal vein. Finally, incorporation of TM within a lipid bilayer accelerates the association of protein C, thrombin and TM, decreasing K_m, and, thereby, significantly increasing the catalytic efficiency of protein C activation (51).

It is noteworthy that the biological activity of liposomal TM is shorter than that reported for detergent solubilized TM injected intravenously into rabbits ($t_{1/2\alpha}$ 12 min; $t_{1/2\beta}$ 460 min) or a recombinant construct of TM that does not contain either transmembrane or intracellular domains when administered intravenously in normal human volunteers ($t_{1/2\alpha}$ 4 hr.; $t_{1/2\beta}$ 20 hrs) (21,52). A shorter half-life likely reflects differences in source, purification and animal model, in addition to the effects of a liposomal formulation that promote localization to the RES. We postulate that TM targeting to the liver provides an optimal strategy for generating high local concentrations of APC at the site of intraportal islet transplantation. However, further study will be required to confirm that reconstitution within liposomes reduce dosing requirements when compared to systemically administered soluble TM.

Conclusions

Primary islet nonfunction due to an IBMIR leads to an increase in donor islet mass required to achieve euglycemia. In this study, we have demonstrated that liposomal formulations of TM significantly enhance intraportal engraftment of allogeneic islets through a reduction in local thrombotic and inflammatory processes. Both fibrin deposition and neutrophil infiltration, as well as expression of proinflammatory cytokines were significantly reduced. Moreover, thrombotic responses mediated by human islets in contact with human blood were also reduced by this approach. As an enzyme-based pharmacotherapeutic, this strategy offers the potential for local generation of APC at the site of islet infusion during the initial period of elevated thrombin production.

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