Surface Re-engineering of Pancreatic Islets with Recombinant azido-Thrombomodulin

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Chemoselective conjugation of an azido-functionalized thrombomodulin to pancreatic islets was achieved by Staudinger ligation to a surface-bound bifunctional poly(ethylene glycol) linker. The presence of the tethered thrombomodulin resulted in a significant increase in the production of activated protein C with a reduction in islet-mediated thrombogenicity. This report highlights the potential of tissue-targeted chemistry to reduce donor cell mediated procoagulant and proinflammatory responses.

Type 1 diabetes results from the autoimmune destruction of insulin-producing pancreatic beta cells and inevitably leads to dependence upon exogenous insulin for control of blood glucose. The transplantation of donor human islets into the liver of diabetic recipients is a promising treatment strategy, but remains limited by a high rate of early islet destruction due to the instant blood-mediated inflammatory reaction (IBMIR) triggered by the interaction of infused islets with blood (*1–3*). IBMIR is initiated by the activation of the coagulation and complement systems, which promotes rapid leukocyte infiltration of the donor islet graft (*4*). Heparin, low-molecular-weight dextran sulfate, melagatran, and activated protein C have been used to minimize IBMIR (*5–8*). Systemic delivery of these anticoagulants at therapeutic doses, however, substantially increases the risk of bleeding. We have postulated that biochemically re-engineering the islet surface with constituents that serve to inhibit the activation of the coagulation cascade would provide a novel strategy to reduce IBMIR (Figure 1A). Specifically, we have developed a scheme to conjugate human recombinant thrombomodulin (*r*TM) to the islet surface through bifunctional poly(ethylene glycol) (PEG) linkers in a chemo-and bioorthogonal fashion (Figure 1B). Thrombomodulin (TM), an endothelial cell transmembrane protein, binds thrombin and inactivates its known procoagulant–proinflammatory properties (*9*). In addition, the TM–thrombin complex rapidly activates circulating protein C (APC), which inactivates clotting factors **Va** and **VIIIa**, thereby serving as a negative feedback system by reducing new thrombin generation (*10, 11*). In this regard, re-engineering islet surfaces with TM and localizing the activation of protein C may provide a novel strategy to reduce IBMIR.

Recent innovations in protein engineering and bioorganic synthesis facilitate incorporation of special tags into recombinant proteins for site-specific modification and immobilization through selective chemical reactions that are orthogonal to the diverse functionality of biological systems (*12*). For example, using a biosynthetic approach, we have recently created a short TM construct containing the thrombin and protein C binding

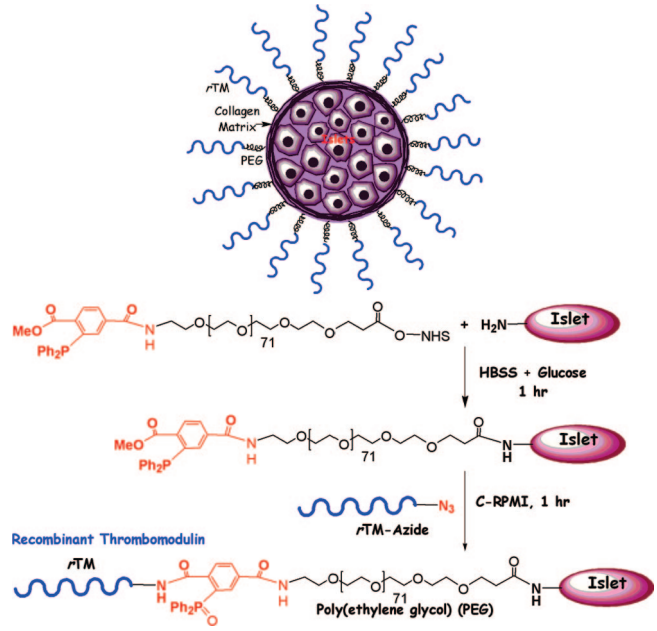

Figure 1. PEGylation and subsequent Staudinger ligation between recombinant azido-thrombomodulin and the PEG–phosphine linker.

regions and terminated with an artificial "non-natural" amino acid, azido (N_3)-alanine, at the C-terminus of the protein (*13*). The azide has secured a prominent role as a unique chemical handle for bioconjugation. We have made extensive use of the azide as a chemical handle for site-specific PEGylation in solution via Staudinger ligation and immobilization of proteins on artificial surfaces via azide-alkyne [3 + 2] cycloaddition (*13, 14*). The Staudinger ligation is particularly advantageous, as it can be performed in living systems without physiological harm, in high yield, and within short time periods offering the potential to re-engineer a variety of cell and tissue surfaces (*15*).

PEG has been widely used to functionalize solid surfaces for inert and biocompatible surface engineering, as well as to limit immunogenicity and prolong the circulating half-life of proteins and peptides for drug delivery. Recently, it has been suggested that PEG grafted onto the collagen matrix of isolated islets could inhibit pathways leading islet xenograft rejection (*16*). Specifically, PEGylation leads to a reduction in antibody/complement-mediated cytotoxicity (*16*). Under this premise, we utilized a

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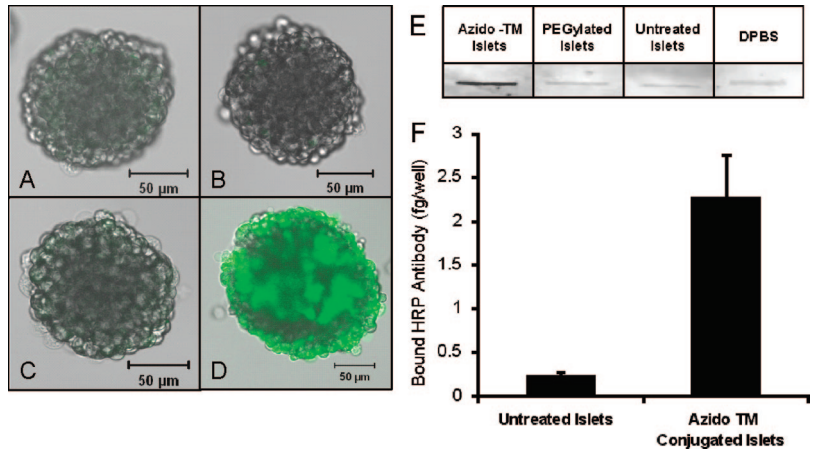

Figure 2. Confocal images (10×) after staining with anti-S-tag antibody of (A) native mouse islets; (B) islets treated with PEG linker; (C) azido-thrombomodulin; or (D) both PEG and azido-thrombmodulin. (E) Slot blot of cell lysate of mouse islets stained with sheep antihuman thrombomodulin antibody, followed by an antisheep HRP. (F) ELISA assay using antihuman thrombomodulin–HRP antibody to measure islet-bound human thrombomodulin. Data points represent the mean ± SD, for a minimum of three independent measurements.

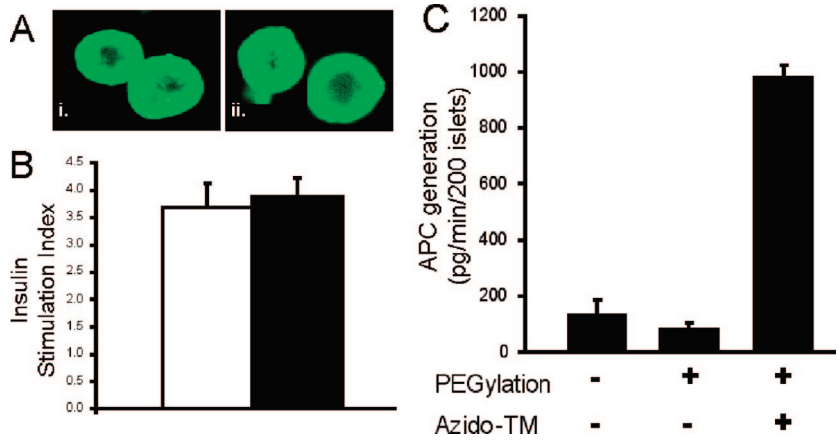

Figure 3. (A) Confocal images (10×) of untreated (i) and TM-conjugated islets using live/dead staining assay (ii). (B) Insulin stimulation index for native (open bars) and thrombomodulin-treated (solid bars) islets. Data points represent the mean ± SD, for a minimum of six independent measurements ($p > 0.05$). (C) Activated protein C (APC) generation by native islets or those treated with PEG linker alone or both linker and azido-TM. Data points represent the mean ± SD, for a minimum of three independent measurements (*$p < 0.05$).

bifunctional PEG linker to chemoselectively target recombinant azido-TM to the surface of living islets in a two-step process with the goal of creating a localized anti-inflammatory environment and reducing islet thrombogenicity.

The bifunctional PEG linker, with a triphenylphosphine (phosphine-PEG-NHS) at one end and a succinimidyl active ester at the other, was synthesized from commercially available amino-PEG-carboxylic acid, as described in Supporting Information. Pancreatic islets were isolated and purified from B10.BR mice using standard techniques by intraductal injection of rodent collagenase (Sigma, MO) (*17*). After culturing islets for 48 h, islets were transferred to a 12 μm plate insert (Millipore, MA) within a 24 well plate. RPMI media was flushed from the islets using PBS (Mediatech, VA). Following washes, the mouse islets were incubated for 1 h with 4 mM phosphine-PEG-NHS in PBS supplemented with 11 mM glucose solution. After a PBS wash, the islets were incubated for 1 h with recombinant azido-TM in fully supplemented RPMI. To elucidate the specificity of the reaction, confocal imaging, slot blot analysis, and ELISA were performed on several test groups, including (i) islets receiving no treatment; (ii) islets undergoing PEGylation alone; (iii) islets incubated solely with azido-TM; and (iv) islets treated sequentially with PEGylation and azido-TM. Since the recombinant azido-TM was expressed as fusion protein bearing a unique N-terminal S-Tag, a FITC-labeled anti-S-Tag antibody was used to detect islet bound TM (Figure 2).

The sequential two-step reaction with both linker and TM led to significant islet labeling, which was not observed on islets exposed solely to azido-TM or PEG linker alone (Figure 2A–D). Slot blot analysis of cell lysates confirmed the presence of human recombinant TM only on mouse islets treated with both linker and azido-TM (Figure 2E). Moreover, an ELISA assay performed with a horseradish peroxidase (HRP) conjugated antihuman TM antibody demonstrated a 10-fold increase in antibody binding to remodeled islet surfaces when compared to untreated islets or to those treated with linker alone ($p < 0.05$; Figure 2F).

Confocal fluorescence imaging using a live/dead staining protocol confirmed preservation of islet viability after surface treatment with linker and Staudinger ligation of azido-TM (Figure 3A). Likewise, glucose-stimulated insulin secretion, as a measure of islet function, was unchanged after surface treatment (Figure 3B). Significantly, a 7-fold increase in protein C activation was observed for islets conjugated with the recombinant azido-TM compared to untreated or pegylated islets (Figure 3C). Moreover, TM conjugation substantially delayed clot formation upon incubation of untreated islets in human plasma. The calculated half-maximal clotting times were 8.15 ± 0.67 min for untreated islets, 9.88 ± 0.49 min for pegylated islets, and 14.08 ± 2.07 min for TM-treated islets ($p < 0.05$).

To date, traditional coating techniques have focused on passive barrier methods that either immunoisolate or immuno-

camouflage transplanted cells from the host. The generation of a bioactive coating through tissue-targeted chemistry serves not only to mask cell surface proteins, but also to provide a means to alter the local microenvironment of the islet graft. In the process, islet-mediated procoagulant and proinflammatory responses are reduced, which may lead to a substantial improvement in clinical outcomes. In principle, the chemical scheme illustrated in this report highlights the potential to immobilize a wide array of functionally complex constituents onto the islet surface in order to actively manipulate the donor–host tissue interface.

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Supporting Information Available: Detail synthetic procedures and spectral data for intermediates and final PEG linker, procedures for islet isolation, assays for islet viability, function, APC generation, and in vitro clotting time (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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