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Sphingosine-1-phosphate improves endothelialization with reduction of thrombosis in recellularized human umbilical vein graft by inhibiting syndecan-1 shedding *in vitro*

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ABSTRACT

Sphingosine-1-phosphate (S1P) has been known to promote endothelial cell (EC) proliferation and protect Syndecan-1 (SDC1) from shedding, thereby maintaining this antithrombotic signal. In the present study, we investigated the effect of S1P in the construction of a functional tissue-engineered blood vessel by using human endothelial cells and decellularized human umbilical vein (DHUV) scaffolds. Both human umbilical vein endothelial cells (HUVEC) and human cord blood derived endothelial progenitor cells (EPC) were seeded onto the scaffold with or without the S1P treatment. The efficacy of recellularization was determined by using the fluorescent marker CellTracker CMFDA and anti-CD31 immunostaining. The antithrombotic effect of S1P was examined by the anti-aggregation tests measuring platelet adherence and clotting time. Finally, we altered the expression of SDC1, a major glyocalyx protein on the endothelial cell surface, using MMP-7 digestion to explore its role using platelet adhesion tests *in vitro*. The result showed that S1P enhanced the attachment of HUVEC and EPC. Based on the anti-aggregation tests, S1P-treated HUVEC recellularized vessels when grafted showed reduced thrombus formation compared to controls. Our results also identified reduced SDC1 shedding from HUVEC responsible for inhibition of platelet adherence. However, no significant antithrombotic effect of S1P was observed on EPC. In conclusion, S1P is an effective agent capable of decreasing thrombotic risk in engineered blood vessel grafts.

Statement of Significance

Sphingosine-1-phosphate (S1P) is a low molecular-weight phospholipid mediator that regulates diverse biological activities of endothelial cell, including survival, proliferation, cell barrier integrity, and also influences the development of the vascular system. Based on these characters, we the first time to use it as an additive during the process of a small caliber blood vessel construction by decellularized human umbilical vein and endothelial cell/endothelial progenitor. We further explored the function and

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mechanism of S1P in promoting revascularization and protection against thrombosis in this tissue engineered vascular grafts. The results showed that S1P could not only accelerate the generation but also reduce thrombus formation of small caliber blood vessel.

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1. Introduction

Sphingosine-1-phosphate (S1P) is a low molecular-weight phospholipid mediator that regulates diverse cellular functions including cell barrier integrity [1–5]. Platelet [1], HDL [6] and lymphocyte [7] are pools of bioactive S1P. Circulating S1P acts as a mediator and leads to responses in endothelial cells (EC) including survival, proliferation, and also influences the development of the vascular system [2,8,9]. Previous studies in our lab revealed that S1P induces VEGF-C expression in endothelial cells through the MMP2/FGF-1/FGFR-1 pathway [10] and promotes c-Src activation, which lead to PECAM-1 phosphorylation [11].

S1P not only regulates a wide array of biological activities in EC but is also maintains EC barrier function. EC is covered by a glycocalyx layer at the blood-tissue interface. A layer of glycocalyx covered on EC that exert a series of biological functions such as keeping vascular permeability and signal transmission [12]. The glycocalyx consists of glycoproteins and proteoglycans with glycosaminoglycan side chains. The most important glycosaminoglycans on EC include heparan sulfate, chondroitin sulfate, and hyaluronic acid. Glypican-1 and syndecan-1 (SDC1) are two major heparan sulfate proteoglycans on EC with potent antithrombotic action [13]. Recent *in vitro* data suggested that S1P contributed to the protection of glycocalyx via the S1P₁ GPCR and prevented heparin sulfate, chondroitin sulfate, and the SDC1 shedding caused by metalloproteinases [14]. A study using rat mesenteric microvessels showed that S1P played a crucial role in stabilizing the glycocalyx of EC and the maintenance of normal vascular permeability [15]. Syndecan-1 shed from EC was one of the components in the thrombi found in animals with bacterial infection. SDC1 aggregated with vWF, fibrin, fibronectin, and also bacteria, activated platelets, and leukocytes causing thrombi occluding the lumen of vessels that could lead to sudden death [16]. According to these relevant biological actions of S1P, it may therefore be useful in construction of tissue engineered vessels where thrombosis is a major complication.

Bypass grafting using autologous small caliber vascular grafts (<6 mm) such as the internal mammary artery, saphenous vein, and radial artery has been developed into a routine procedure with good clinical outcomes. However, there are still a significant number of patients who cannot benefit from this procedure because of the limited availability of healthy autologous vessels due to pre-existing venous diseases or trauma. The use synthetic and engineered biological materials as vascular grafts under certain conditions is gaining acceptability [17]. However, thrombosis and compliance mismatch have limited the application of these materials to generate grafts less than 6 mm in diameter as a vascular substitute [18–20].

Many studies have shown that human umbilical vein (HUV) can be used as a living scaffold by different experimental approaches. The cross-linked HUV with internal diameters from 4 to 6 mm is functionally similar to arteries that used as dialysis shunts and for peripheral artery reconstructions for over 40 years [21,22]. The decellularized human umbilical vein (DHUV) has been verified to provide satisfactory mechanical properties as a scaffold for cardiovascular tissue engineering after proper dissection [23]. Furthermore, it has been suggested that cell seeding of DHUV

scaffolds may restore otherwise compromised properties of the engineered vessel [24].

Endothelial cells (EC) line the interior surface of blood vessels, including arteries, veins and capillaries, and represent an important regulator of blood coagulation [25]. Endothelial progenitor cells (EPC) have the ability to differentiate into EC in the peripheral circulation. they ultimately homing to regions of blood vessel formation [26–29]. EPC are isolated easily and have potential to be applied in blood vessel construction [30–34]. S1P is found to induces the migration and angiogenesis of EPC through the S1PR3/PDGFR-beta/Akt signaling pathway [35].

As an allogeneic vessel using HUV, the immunoreaction induced by cellular components is eliminated. However, the lack of mature EC represents the loss of an important antithrombogenic function. Therefore, recellularization is a necessary procedure for replacement of the vascular EC lining. However, current recellularization methods are time consuming and ineffective. Base on the effects of S1P on EC and EPC, we explored the function and mechanism of S1P in promoting revascularization and protection against thrombosis in this tissue engineered vascular grafts. Our result showed that S1P enhanced the attachment of HUVEC and EPC. Based on anti-aggregation tests, S1P-treated HUVEC recellularized vessels when grafted showed reduced thrombus formation compared to controls. Our results also identified reduced SDC1 shedding from HUVEC responsible for inhibition of platelet adherence.

2. Materials and methods

EC and EPC were seeded on the DHUV by rotational method separately with and without the presence of S1P. The antithrombotic character of these four types of tissue engineered blood vessel was compared with native HUV and DHUV using anti-aggregation tests measuring platelet adherence and clotting time. The action of S1P was further explored by first digesting SDC1 on HUVEC with MMP-7 followed by treating the cells with S1P, and also by using these two treatments in the reverse order. These effects were evaluated by counting the number of platelets attached to the treated HUVEC.

2.1. Ethics assurance

Human umbilical cords and cord blood were harvested in the Department of Obstetrics of the Taipei Veterans General Hospital (Taiwan) with informed consent signed by the donors. The entire procedure was performed in accordance with governmental regulations (Guidelines for Collection and Use of Human Specimens for Research, Department of Health, Taiwan) and after approval from the Institutional Review Board (Approval number 2013-08-020BC, Taipei Veterans General Hospital, Taiwan).

2.2. Preparation of DHUV

Umbilical veins with approximate lengths of five cm were isolated by removing the arteries and Wharton's jelly. Decellularization was started with a 2-day incubation using 0.1% sodium dodecyl sulfate (SDS; Sigma-Aldrich, St. Louis, MO, USA), followed by 2-day washing with phosphate-buffered saline (PBS; pH 7.4; Gibco, Carlsbad, CA). Medium 199 (Gibco) containing 20% fetal

bovine serum (FBS; Gibco, South America) was used to remove remaining DNA for two days and the vessels were washed with PBS subsequently [36,37]. The decellularization steps were performed at 37 °C on a shaker with high-speed agitation under sterile conditions.

The decellularizing efficiency was examined by hematoxylin and eosin staining (HE; Sigma-Aldrich) and DNA quantification using Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen, Carlsbad, CA, USA) to confirm that more than 95% of the cells was removed and the complete scaffold structure remained intact (Data not shown) [36].

2.3. Isolation, culture, characterization of HUVEC and EPC

HUVEC were isolated from fresh umbilical cords by treatment using 0.1% (w/v) type I collagenase (Sigma-Aldrich) in cord buffer (136.9 mM NaCl, 4 mM KCl, 10 mM HEPES and 11.1 mM glucose, pH 7.65) and incubated at 37 °C for 20 min. The HUVEC were collected by centrifugation (1500g, 5 min) and seeded onto a 10-cm dish coated with 1% gelatin (Sigma-Aldrich) in EGM-2 medium. The EGM-2 medium was made up of EC basal medium (Lonza, Walkersville, MD) supplemented with EGM-2 SingleQuots (Lonza), 2% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were passaged weekly and were subcultured after trypsinization. Passages 2–4 were used in the experiments.

EPC were derived from the buffy coat of human umbilical cord blood obtained by centrifugation at 700g for 20 min and diluted with an equal volume of Dulbecco's phosphate-buffered saline (D-PBS; Sigma-Aldrich). The buffy coat cells were then layered onto Ficoll-Paque solution (1.077 g/ml; Amersham; Uppsala, Sweden) and centrifuged at 700g for 40 min to deplete residual red blood cells, platelets, and plasma. Mononuclear cells (MNC) at the interface were collected and washed twice with D-PBS. The MNCs were seeded at a concentration of 10^6 cells cm^{-2} in EGM-2 medium (Lonza) in fibronectin-coated ($2 \mu\text{g cm}^{-2}$, BD Biosciences, Franklin Lakes, NJ) T25 flasks and cultured at 37 °C in a humidified atmosphere with 5% CO_2 . Nonadherent cells were removed using a medium change after 3 days of seeding and the medium was changed every 3 days thereafter for 2–3 weeks. When well-developed colonies of endothelial-like cells became visible, the cells were washed with PBS, harvested with 0.05% trypsin-EDTA (Gibco) and plated in new T75 flasks.

EPC and HUVEC were identified by surface markers, including human CD14, CD31, CD34, CD45, CD105 (VEGFR2), CD309, and von Willebrand factor (vWF) using a BD Accuri™ C6 Flow Cytometer (Becton-Dickinson, San Jose, CA). A replicate sample was stained with mouse IgG1 antibody as an isotype control to ensure specificity (data not shown).

2.4. Cell staining, seeding on scaffolds, and counting

HUVEC and EPC were stained with CellTracker™ Green CMFDA (Invitrogen, Carlsbad, CA, USA) before starting the recellularization of the vein scaffolds. The staining began by removing the medium from each dish and adding pre-warmed $5 \mu\text{M}$ labeling solution at 37 °C for 30 min. The labeling solution was changed to fresh pre-warmed medium and incubated for an additional 30 min at 37 °C. Finally, the cells were washed with PBS and suspended with culture medium. Five cm DHUV segments were everted with the luminal surface facing outward so that the cells could be seeded on it. And the veins were fixed in the middle of a tube using a glass rod. This The labeled cells were suspended in the culture medium in the presence of $1 \mu\text{M}$ S1P in the culture tube [2,9,11]. The culture tube was rotated around the central axis with 1 rpm at 37 °C for 24 h. Then the cells attached to the HUV were visualized by fluorescence microscopy at 517 nm excitation and the

fluorescent cells were analyzed using the MetaMorph program (Molecular Devices, Sunnyvale, CA). Some of the recellularized grafts were processed for paraffin embedding and stained with anti-Human CD31 (Becton and Dickinson, San Jose, CA) to confirm the attachment of the cells. The vessels seeded with cells were reverted before subsequent experiments.

2.5. Coagulation and kinetic clotting tests

Venous blood samples were drawn from the antecubital vein of healthy volunteers and collected in a 15-ml tube containing 3.8% sodium citrate (Sigma-Aldrich) as anticoagulant at a 1:9 ratio of sodium citrate/blood. The blood was injected into the vessels containing HUVEC or EPC in the presence or absence of $1 \mu\text{M}$ S1P. Non-cell-coated DHUV and Eppendorf tubes were served as controls. To initiate the blood coagulation cascade, 0.25 M calcium chloride solution was added to the citrated blood samples. The two ends of the vessels which were approximately two cm in length were then sealed and after a predetermined time, one end of each vein was cut, and the blood sample was transferred into a 15-ml tube containing 5 ml of distilled water. Red blood cells were broken up by the hypotonic solution and released hemoglobin. The red blood cells that had not been trapped in a thrombus were hemolyzed, whereas free hemoglobin was dissolved in the water. The concentration of the free hemoglobin dissolved in the water was colorimetrically measured at 540 nm wavelength using a plate reader. The change in the optical density of the solution versus time was plotted. Clotting times were estimated for all test materials, including the tubes, non-seeded HUV, cell-seeded HUV and cell-seeded HUV with S1P treatment as previously described [38,39].

2.6. Platelet adhesion test

Platelet-rich plasma (PRP) was obtained from healthy donors by centrifugation at 200g for 20 min at room temperature without braking. Approximately two-thirds of the PRP was transferred into a new plastic tube and centrifuged at 100g for 20 min at room temperature without braking to pellet contaminating red and white blood cells. The supernatant was again transferred into new plastic tube and centrifuged at 800g for 20 min at room temperature without braking to pellet platelets. The CD62 positive platelet was about 40% in the PRP separated by a similar method [40]. The pellet was suspended in D-PBS at a concentration of $1.0 \times 10^9 \text{ ml}^{-1}$. Native, decellularized and recellularized vessels approximately two cm in length were tested. The recellularized vessels were prepared by HUVEC or EPC seeded DHUV in medium with or without $1 \mu\text{M}$ S1P supplementation. The six grafts were immersed in PRP and incubated at 37 °C for 1 h and subsequently rinsed with a 0.9% NaCl solution (Sigma-Aldrich) to remove weakly adherent platelets. Next, the adherent platelets were fixed with 2.5% glutaraldehyde solution (Sigma-Aldrich) at room temperature for 16 h. The samples were washed three times for 10 min each with D-PBS and then treated with 1% osmium tetroxide (Sigma-Aldrich) for 1 h. After treatment, the samples were rinsed with D-PBS three times before embedding for scanning electron microscopic (SEM) examination. The specimens were coated with a 10–20 nm-thick gold layer after critical point drying and examined using SEM. Ten fields at 2000x magnification were chosen at random to obtain high-confidence statistics to quantify adherent platelets.

2.7. Manipulation of SDC1 expression in recellularized vessels

2.7.1. Effect of S1P on SDC1 expression

EC and EPC were prepared as above described and seeded on ethanol-disinfected 18 mm round coverslips (Biomaterial Scientific

Co., Ltd, Taiwan) placed in 12-well plates. 2×10^6 cells well⁻¹ were applied and incubated at 37 °C, 5% CO₂ for 1 h before the FBS-free culture medium was added for incubation overnight. Next morning, medium containing 1 μ M S1P and 0.1% fatty-acid free bovine serum albumin (FAF-BSA; Sigma-Aldrich) was added for 6 h. The coverslips were stained with anti-SDC1 (Abcam, UK) and anti-Glypican-1 antibodies (Genetex, USA) using Alexa Fluor 647 goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA, USA).

For immunofluorescence staining, the cells were fixed with 2% paraformaldehyde and blocked by 5% BSA (BIOMAN, Taiwan) in PBS. Glycocalyx shedding was imaged with a Zeiss AxioPlan 2 fluorescence microscope and quantified with image J.

2.7.2. The reaction of MMP-7 on SDC1

MMP-7 (Abcam, UK) is a proteinase that can specifically cleave SDC1 [41]. In order to determine the proper digestion time of MMP-7 for the removal of SDC1, a series of time points was selected ranging from 1 h to 24 h. The vessels were subsequently fixed and processed for immunofluorescence staining using an anti-SDC1 antibody and quantified with image J. Based on these results, 6 h was selected as an optimal time point that was sufficient for the near complete removal of SDC1 by MMP-7.

2.7.3. Platelet adhesion to EC treated with S1P and/or MMP7

2.7.3.1. Platelet staining. The freshly isolated platelets were adjusted to 2×10^9 ml⁻¹ in D-PBS. Sudan Black B (Sigma-Aldrich) solution was prepared in 70% ethanol at a concentration of 10 mg ml⁻¹ and filtered through 0.22 μ m filter. This staining solution was added at a volume ratio of 1:20 to the platelet suspension for 30 min at room temperature [42]. After staining, the platelets were washed with D-PBS three times and suspended in EC culture medium at 1×10^{10} ml⁻¹.

2.7.3.2. The influence of S1P and MMP-7 treatment on platelet adhesion to EC. EC was seeded at 2×10^5 per well in 200 μ l in 8-well slide chambers (Nalge Nunc, USA) and treated separately with either: (1) 0.1% FAF-BSA for 4 h; (2) 1 μ M S1P for 4 h; (3) 1 ng ml⁻¹ MMP-7 for 6 h; (4) 1 ng ml⁻¹ MMP-7 for 6 h followed by 0.1% FAF-BSA for 4 h; (5) 1 ng ml⁻¹ MMP-7 for 6 h followed by 1 μ M S1P for 4 h; (6) 0.1% FAF-BSA for 4 h followed by 1 ng ml⁻¹ MMP-7 for 6 h; (7) 1 μ M S1P for 4 h followed by 1 ng ml⁻¹ MMP-7 for 6 h; respectively. After changing to culture medium, the Sudan black B-stained platelets were added to the cells for 1 h at 37 °C and 5%

CO₂ with gentle shaking at 20 RPM on the orbital shaker (OS701, DEAGLE, Taiwan). Subsequently, the slides were gently washed by D-PBS and stained with HE. The slides were observed under a Zeiss Axio Observer A1 light microscope at 400x magnification and 7 fields from each treatment were randomly chosen to count the platelets attached to the cells. The data was analyzed using one-way ANOVA and the experiment was repeated three times.

2.8. Statistical analysis

All experiments were repeated at least three times and the data shown as the mean $\pm$ SD. Mann-Whitney *U* test was applied for statistical analysis between experimental groups where $P < 0.05$ were considered significant. The Kruskal-Wallis test with post-hoc Mann-Whitney *U* test was used to compare data more than two groups where $P < 0.05$ were considered significant. Statistical analysis was performed using IBM SPSS Statistics 19 (version 19; SPSS, Chicago, Illinois).

3. Results

3.1. S1P promotes cell attachment to decellularized DHUV

After the cells were seeded onto the decellularized HUVE for 24 h in culture, the recellularized DHUV was examined using fluorescence microscopy, and cell attachment was quantified by the MetaMorph program. One μ M S1P significantly ($p < 0.05$) enhanced HUVEC attachment compared to the non-treated controls (Fig. 1A). A similar enhancement by S1P was also observed for EPC (Fig. 1B, $p < 0.05$). These results indicated that S1P can enhance both HUVEC and EPC attachment to the decellularized HUVE scaffolds.

3.2. Cellular characteristics of the recellularized DHUV

Neo-epithelial layers in recellularized DHUV were stained with anti-human CD31. CD31 expression was well visible in the neo-epithelial layer formed by HUVEC or EPC (Fig. 2). We confirmed that the HUVEC and EPC had attached to the inner layer of the DHUV. Compared with the results of DAPI staining, the expression of CD31 on HUVEC and EPC reached similar levels after S1P treatment.

Fig. 1. S1P increases recellularization of DHUV. Panel A, Attachment of HUVEC after rotational seeding to DHUV with or without 1 μ M S1P treatment were calculated by the MetaMorph program; Panel B, Attachment of EPC after rotational seeding to DHUV with or without 1 μ M S1P treatment were calculated by the MetaMorph program. Note that the cell numbers following S1P treatment were significantly higher than that of the controls. The counts represent the mean $\pm$ SD from at least three independent experiments. * $P < 0.05$.

Fig. 2. Immunofluorescent staining for CD31 marker positive cells in recellularized scaffolds. S1P applied at 1 μ M increased the attachment of HUVEC and EPC to the inner vascular wall of a DHUV. Normalized to DAPI positive cell number, the expression of CD31 in HUVEC and EPC maintained a similar level. Original magnification, 200X; scale bar = 50 μ m.

3.3. Recellularization in the presence of S1P reduces thrombogenesis in DHUV

We also determined blood clotting profiles on the various recellularized surfaces (Fig. 3). In this assay, blood clotting sharply decreases absorbance at 540 nm (A540) in the solution. This

Fig. 3. Kinetics of blood coagulation on different matrices. The blood clotting time was measured in recellularized vessels seeded with cells treated with or without S1P. The clotting time of S1P-treated HUVEC was significantly longer than in the others. The data represent the mean $\pm$ SD of three independent experiments. * $P < 0.05$, *** $P < 0.01$.

phenomenon can be used to trace the time course of blood clotting. The absorbance value at the starting point was used as the baseline, corresponding to no clot formation in the vessel. The A540 of distilled water, was 0.01, was regarded as hemoglobin free condition and the time required to reach this A540 value was defined as the clotting time. Thus, a lower A540 due to more platelets being locked into the clot indicated more thrombus formation. The A540 values of tubes and non-cell-seeded DHUV started decreasing at 15–20 min and the blood samples could no longer be gathered after 25 min. In contrast, the A540 values of the HUVEC-seeded DHUV and the EPC-seeded DHUV did not decrease for 35–40 min. These results demonstrated that the clotting time of the recellularized DHUV was much longer than that of the DHUV. The clotting time of S1P-treated HUVEC-seeded DHUV was significantly longer than that of untreated DHUV. However, there was no statistically significant difference between the non-S1P-treated control and S1P-treated EPC-seeded DHUV. This finding suggested that S1P treatment had no effect on the anti-thrombotic properties of EPC; whereas, S1P significantly enhanced the antithrombotic properties of HUVEC-recellularized HUV.

Platelet adhesion was also performed to analyze the thrombogenicity of these types of vessels under an SEM (Fig. 4). After HUV was incubated with PRP for 1 h, nearly no platelets were attached to the EC-covered area of native untreated HUV (2.8 ± 1.23 field $^{-1}$), whereas several platelets were observed on the EC-free area. In contrast, the DHUV cross-sections under the SEM showed roughness surface on which were covered by many platelets. Recellularized DHUV showed a significantly decreased number of adherent platelets per field compared to the DHUV and, consequently, revealed evidence for lower thrombogenicity of the recellularized DHUV (Fig. 4A). Statistical analysis demonstrated that the number of platelets that adhered to HUVEC (8.0 ± 3.43 field $^{-1}$) or EPC-seeded DHUV (5.3 ± 3.02 field $^{-1}$) was significantly lower than the number that adhered to DHUV (40.9 ± 6.77 field $^{-1}$) (Fig. 4B–D). Furthermore, S1P treatment

Fig. 4. SEM of the luminal surface of vessels incubated with PRP for 1 h. Panel A, Only a few platelets adhered at the area of the cell junctions in the HUUV (white arrows). In contrast, groups of platelets attached to the decellularized vessel scaffold. The cross-section of DHUV after seeding with HUVEC for 24 h in the presence of S1P showed that fewer platelets adhered to the scaffold than the control vehicle treated graft. In DHUV seeded with HUVEC for 24 h in the presence of S1P platelets adhered to the scaffold as much as seen in the vehicle treated control. The scale bar is 10 μ m. Panels B, C, D, Quantification of platelet adhered to the luminal surface of DHUV. Panel B, The number platelets adhered to the DHUV is significantly higher than to native HUUV. Panel C, Compare to DHUV seeded with HUVEC in normal culture medium attachment in S1P treated HUVEC vessels was significantly lower than the controls. Panel D, The number of platelets attached to S1P treated EPC seeded DHUV showed no difference over vehicle controls. Original magnification, 2000X; scale bar = 10 μ m * P < 0.05, *** P < 0.001, n = 10.

significantly decreased the number of platelets that adhered to HUVEC-seeded DHUV (1.4 ± 1.7 field⁻¹) compared with non-treated controls (8.0 ± 3.43 field⁻¹) (Fig. 4C). However, the effect of S1P treatment on platelet adhesion to EPC-seeded DHUV was not significant compared with non-treated controls (6.6 ± 3.24 field⁻¹ VS 5.3 ± 3.02 field⁻¹) (Fig. 4D).

3.4. The effect of S1P on the glycocalyx expression in EC and EPC

Next, we investigated the mechanisms underlying the antithrombotic effects of S1P on EC. Two important proteoglycans, Glypican-1 and SDC1 were evaluated [13]. Although Glypican-1 expression was detectable on HUVEC and EPC, no difference was observed after S1P treatment (Supplemental Fig. 1). In contrast, expression of SDC1 could readily be observed on HUVEC. However, SDC1 expression was low on the surface of EPC. When EC and EPC were treated 1 μ M S1P, the expression of SDC1 on HUVEC was considerably higher than in FAF-BSA controls.

In contrast, S1P had no effect on SDC1 expression in EPC (Fig. 5A–C). After treatment with MMP-7, the expression of Glypican-1 on EC remained at control level (Supplemental Fig. 2). These results indicated that MMP-7 did not have reaction with Glypican-1. Thus, we focused on the function of S1P on SDC1 expression in HUVEC.

3.5. S1P protects SDC1 on EC and further prevents platelet adhesion

Immunofluorescence staining by anti-SDC1 showed that the percentage of SDC1 immunoreactivity on HUVEC decreased with time (Supplemental Fig. 3). We determined that the optimal time for MMP-7 digestion of SDC1 in HUVEC was 6 h. When the platelets were added to cells treated with S1P with or without MMP-7 digestion and incubated for 1 h, the number of platelet deposits on S1P-treated-HUVEC was significantly less than in FAF-BSA treated controls (68 ± 15 vs. 146 ± 37 , p < 0.001). In contrast, there were more platelets attached to MMP-7 treated cells averaging 200 ± 64 (Fig. 6A). This finding is consistent with the hypothesis

Fig. 5. Syndecan-1 expression in cells treated with S1P. Panel A, In HUVEC treated with 1 μ M S1P expression of Syndecan-1 increase relative to FAF-BSA vehicle controls. However, no such change was visible in EPC. Original magnification, 400X; scale bar = 50 μ m; Panel B, Fluorescence intensity in the cells showed higher Syndecan-1 expression in S1P treated HUVEC over vehicle controls. Panel C, Fluorescence intensity in EPC treated with S1P showed no difference with solvent control. * $P < 0.05$, $n = 10$. The fluorescent intensity of S1P treatment group was normalized to control group in each test.

that SDC1 was cleaved by MMP-7 promoting the adherence of platelets to HUVEC (see Fig. 7).

In order to investigate the protective function of S1P, most of SDC1 on the surface of HUVEC had to be removed by MMP-7 after a 6-h treatment. At this time point, fresh medium containing 1 μ M S1P of FAF-BSA control was added to the cells followed by the addition of platelets. These result of platelet adherence showed that there were fewer platelets attached to the cells compared to the FAF-BSA control group (56 ± 16 vs. 192 ± 27 , $p < 0.001$, Fig. 6B). Using HUVEC pretreated with S1P for 4 h and subsequently digested with MMP-7 for 6 h, the number of platelets attached to the cells showed no difference relative to the FAF-BSA control (349 ± 63 vs. 439 ± 97 , Fig. 6C).

4. Discussion

In the present report, we provide evidence that the small molecule bioactive lipid S1P when applied to DHUV together with EC and EPC under dynamic coating conditions enhances the development of a confluent functional endothelial surface. In the case of EC seeding but not EPC seeding, the S1P-augmented endothelium has significantly enhanced antithrombotic surface properties due to reduced SDC1 shedding. Thus, S1P presents a new potential intervention to generate engineered vascular grafts *in vitro* that mimic the antithrombotic surface properties of normal vessels.

Autologous vessels, synthetic materials, and biomaterials have been used as small-caliber vascular grafts in patients. All grafts, especially small-caliber synthetic bypass below 6 mm, face the problems of thrombosis, immune rejection, and biodegradation [43]. Although there is no relevant difference according to patency rates between the materials used at present in cardiovascular patients, it has been shown in several *in vivo* trials that synthetic

grafts are inferior to autologous substitutes [44]. Especially for long term applications the patency rates are much lower with nearly 70% (71% of PET and 74% of ePTFE) after one year and 58% (59% of PET and 56% of ePTFE) after three years compared to 90% and 81% for autologous prostheses, respectively [43,45–48]. Here we used DHUV to prevent immunorejection. Endothelium on the luminal surface of blood vessels plays important roles in preventing thrombosis. In our study, both HUVEC and EPC were used for coating DHUV with the objective of producing a confluent, functional antithrombotic surface. HUVEC are mature EC that line HUV whereas, EPC can be differentiated into EC and easily be harvested from peripheral blood of patients [38,49]. Generation of an engineered vascular graft usually takes more than 8 weeks. Generally, in cell-seeding experiments, the seeding surface is pre-coated to improve cell adhesion. Several biomolecules have been used to pre-coat bypass grafts to enhance EC attachment. Herring et al. [50] coated vascular prostheses with various extracellular matrix proteins and blood products to enhance EC retention. Extracellular matrix components including fibrin [51], collagens [52–54], elastin, fibronectin [53–55], laminin [56], and aptamers [57] are often used to pre-coat tissue-engineered grafts. However, pre-coating has the limitation that the coating is washed-off when exposed to arterial blood flow. Type II monocytes can also be used to accelerate the formation of a functionally-confluent endothelial cell monolayer on polymeric surfaces [58]. To overcome these difficulties, we explored the use of a biomolecule, S1P that can directly enhance EC attachment without pre-coating of DHUV. This procedure eliminated the washing-off of pre-coated molecules during dynamic cell seeding. To our knowledge this is the first report on using a small biomolecule, such as S1P to enhance EC attachment in a tissue-engineered vascular scaffold. Our results show that S1P-treated EC attached better to the DHUV treated under both static and dynamic cell seeding conditions.

Fig. 6. Effect of MMP-7 digestion on attachment of platelets to HUVEC treated with S1P. Panel A, HE staining showed that the number of platelet was fewer in S1P treated HUVEC and was further decreased by MMP-7 treatment. The number of attached platelets increased significantly after MMP-7 treatment in S1P treated HUVEC. Panel B, HE staining showed that the number of platelets attached to HUVEC was fewer when treated with S1P treatment was followed by MMP-7 digestion. The number of platelet was significantly fewer in S1P treated HUVEC which were pre-treated with MMP-7 compared to vehicle control. Panel C, HE staining and statistical analysis showed that the number of platelets attached to MMP-7 digested HUVEC pre-treated with S1P were not different from FAF-BSA pretreated controls. The number of platelet attached was significantly fewer in S1P treated HUVEC and more following MMP-7 treatment of the vessels. The platelets per vision field was presented as Mean $\pm$ SD. The area of vision field was about 2 mm². Original magnification, 400X; Scale bar = 40 μ m. * P < 0.05, *** P < 0.001, n = 7.

An *in vivo* test of using the decellularized small caliber blood vessel for implantation showed that acute thrombosis in the first 24 h cause is the primary blood vessel failure [59]. Thus, a confluent endothelial layer is important for graft patency. The formation of thrombi on the inner surface of a blood vessel correlates with its surface area; larger surface areas are more likely to induce activation of coagulation factors. Kinetic clotting time and platelet

Fig. 7. Scheme of the proposed mechanism of S1P-induced prevention of platelet adhesion via reduced Syndecan-1 shedding from EC. See text for details.

adhesion assays were used to determine the coagulation status of different surfaces that included non-cell-seeded DHUV, HUVEC-seeded UV, and EPC-seeded DHUV. The non-cell-seeded DHUV group formed clots fastest. The rough surface of the DHUV enhanced platelet adhesion and blood clotting confirmed by SEM. Clot formation in the HUVEC-seeded DHUV and EPC-seeded DHUV groups was significantly prolonged, which suggested that the cells seeded on the surface provided an antithrombotic effect. Both EC- and EPC-seeded DHUV showed antithrombotic properties similar to that of native vessels.

Furthermore, the S1P-treated HUVEC showed a significant increase in anti-thrombogenicity. However, such enhancement was not observed in the EPC group treated with S1P. The detailed mechanism underlying the antithrombotic effect remains unclear. Nonetheless, our results point to the role of SDC1 in S1P-induced antithrombosis of EC-coated grafts. Previous studies have established that inflammation causes SDC1 shedding which in turn enhances thrombosis [60,61]. Our results demonstrate that S1P promotes SDC1 expression on EC and SDC1 expression correlates directly with the number of platelets adhering to EC. The findings presented here indicate that a brief 4-h S1P treatment is already sufficient to induce surface SDC1 expression leading to a significant reduction in platelet adhesion. Specific cleavage of SDC1 by MMP7 provided additional evidence for its role in the S1P-induced antithrombotic effect. Taken together, these results suggest that S1P-mediated upregulation of SDC1 is a key element of the reduced thrombosis found in our experiments. Nevertheless, some important variables fibrin degradation, including thrombin-antithrombin III and FD-Dimers level, have not been controlled due to the limitation of our *in vitro* tests, which are difficult to correlate with the *in vivo* coagulation factors. We are planning to monitor these markers in future animal study.

EPC have been shown to induce strong neovascularization when cultured with S1P for 2 h and blood flow recovery after hind limb ischemia in mice [62]. This effect was due to an enhancement of EPC homing to damaged vessels. In our experiments, SDC1 increased in response to S1P treatment of HUVEC but not EPC. S1P significantly suppressed thrombogenesis in a HUVEC-recellularized vessel but not in EPC recellularized grafts. Furthermore, S1P treatment inhibited platelet adhesion to HUVEC monolayers but not to EPC, most plausibly because EPC failed to upregulate SDC1 on their surface. Based on our results, we hypothesize that the lack of S1P effects on EPC might be due to low expression of SDC1. S1P was reported to promote adipose-derived mesenchymal stem cell differentiation to the endothelial lineage with expression of CD31, vWF, and eNOS [63]. The actions of S1P in EPC warrant further investigation. In conclusion, our

results demonstrated that tissue-engineered vessels generated by using decellularized bioscaffold and HUVEC with S1P treatment could represent an effective new approach for generation of functional grafts with reduced thrombogenic properties. The *in vivo* performance of such grafts will have to be evaluated in future studies. The long term function of grafts treated with S1P should also be evaluated in future experiments. Although S1P naturally exists in plasma, the safety and side effects of *ex vivo* treatment should be evaluated before clinical application. We also plan to determine which S1P receptor mediates this effect and use receptor specific agonist drug candidates as an alternative to S1P.

5. Conclusions

Our results demonstrate that tissue-engineered vessels generated from decellularized bioscaffold and HUVEC in the presence of S1P could provide a new clinically relevant approach to construct vascular prostheses. Although S1P seemed to have no effect on EPC, further research could ultimately identify conditions of EPC differentiation to fully mature EC with abundant SDC1 expression. Furthermore, this study suggests that S1P is an effective additive capable of decreasing the thrombotic risk in DHUV seeded with cells of endothelial-lineage. Thus, our report may provide a novel direction for generation of tissue-engineered blood vessels with enhanced anti-thrombogenic properties that is especially important for small-caliber vascular grafts.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.actbio.2017.01.050>.

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