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Substrate-enzyme affinity-based surface modification strategy for endothelial cell-specific binding under shear stress.

Substrate-enzyme affinity-based surface modification strategy for endothelial cell-specific binding under shear stress

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Abstract

Establishing an endothelial cell (EC) monolayer on top of the blood contacting surface of grafts is considered to be a promising approach for creating a hemocompatible surface. Here we utilized the high affinity interactions between the EC plasma membrane expressed enzyme called endothelin converting enzyme-1 (ECE-1) and its corresponding substrate big Endothelin-1 (bigET-1) to engineer an EC-specific binding surface. Since enzymatic cleavage of substrates require physical interaction between the enzyme and its corresponding substrate, it was hypothesized that a surface with chemically immobilized synthetic bigET-1 will preferentially attract ECs over other types of cells found in vascular system such as vascular smooth muscle cells (VSMCs). First, the expression of ECE-1 was significantly higher in ECs, and ECs processed synthetic bigET-1 to produce ET-1 in a cell number-dependent manner. Such interaction between ECs and synthetic bigET-1 was also detectible in blood. Next, vinyl-terminated self-assembled monolayers (SAMs) were established, oxidized and activated on a glass substrate as a model to immobilize synthetic bigET-1 via amide bonds. The ECs cultured on the synthetic bigET-1-immobilized surface processed larger amount of synthetic bigET-1 to produce ET-1 compared to VSMCs (102.9 ± 5.13 vs. 9.75 ± 0.74 pg/ml). The number of ECs bound to the synthetic bigET-1-immobilized surface during 1 h of shearing (5dyne/cm^2) was approximately 3-fold higher than that of VSMCs (46.25 ± 12.61 vs. 15.25 ± 3.69 cells/100xHPF). EC-specific binding of synthetic bigET-1-immobilized surface over a surface modified with collagen, a common substance for cell adhesion, was also observed. The present study demonstrated that using the substrate-enzyme affinity (SEA) of cell type-specific enzyme and its corresponding substrate can be an effective method to engineer a surface preferentially binds specific type of cells. This novel strategy might open a new route toward rapid endothelialization under dynamic conditions supporting the long-term patency of cardiovascular implants.

1. Introduction

Cardiovascular disease is one of the leading causes of death worldwide [1, 2], and severe occlusive vascular disease demands a vascular intervention or a replacement surgery [3]. Auto-grafts, commonly taken from the left internal thoracic artery (LITA) or from the saphenous vein, are the gold standards today for coronary artery bypass grafting (CABG) [4]. However, no suitable veins/arteries are available due to previous use or diseased vein wall in 5-30% of patients [5], and such limited supply of auto-grafts creates a great need for synthetic material-based small diameter vascular prostheses. Unfortunately, synthetic materials *in vivo* often did not support rapid endothelialization and resulted in graft failure, especially in small diameter vascular prostheses (Ø≤4~6 mm) due to thrombosis and the formation of pseudo-intimal hyperplasia followed by restenosis [6, 7].

Formation of a functionally stable endothelial cell (EC) layer on the luminal surface of prosthesis can solve the problems of thrombogenicity and pseudo-intima formation [8-13]. Deutsch et al. as well as Dohmen et al. reported that vascular prostheses pre-seeded with human venous EC showed much longer patency compared to non-seeded ones, strongly emphasizing the importance of establishing a stable EC layer [14, 15]. Nevertheless, laborious preparation steps such as sampling of appropriate cells from patients and pre-seeding of prostheses *in vitro* call for the development of a cell-free, off the shelf vascular prostheses, which can be immediately used for the implantation [16] and recruit EC/EC-precursors from the neighboring tissues and/or from the blood after the implantation. Since the vascular wall and the blood contain heterogeneous cell populations that also contact a polymer surface *in vivo,* finding a way to give a surface EC specificity might be a key element to develop fully functional vascular prostheses. Although establishment of a strongly attached EC layer on top of an artificial surface is a task rather difficult to achieve [17, 18], it is an important requirement for the development of a fully functional small diameter vascular prostheses.

A number of different approaches to engineer hemocompatible surfaces have been tried, and one of them was to modify the surface in a way to mimic the extracellular matrix (ECM) [19-21]. For example, the tripeptide Arg-Gly-Asp (RGD) sequence is a frequently studied ECM constituent peptide sequence promoting cell adhesion via ligand-receptor interaction [22]. The RGD sequence is shared by many different ECM proteins such as fibronectin and collagen, as well as laminin [23, 24]. Other examples of integrin binding peptides to facilitate EC binding are the fibronectin-derived Arg-Glu-Asp-Val (REDV) motif and the osteopontin-derived Ser-Val-Val-Tyr-Gly-Leu-Arg (SVVYGLR) motif [25, 26]. Although all of these peptides demonstrated that they can promote EC adhesion, they did not show sufficient EC-specificity or preferential EC binding over other types of cells. To overcome such problem of insufficient EC-specificity, we introduce a new approach based on substrate-enzyme affinity (SEA) that might provide EC specificity in the present study.

One of the plasma membrane enzymes expressed in EC is endothelin converting enzyme-1 (ECE-1), which is responsible for producing endothelin-1 (ET-1) by cleaving its corresponding substrate big endothelin-1 (bigET-1). According to the induced fit model for enzymes to process corresponding substrates, they form a transition state complex where they specifically recognize and bind to each other [27]. The present study exploits such specific interactions between enzymes and corresponding substrates to create an EC specific surface. In other words, it was hypothesized that a surface with chemically immobilized synthetic bigET-1 will preferentially attract ECs over the other types of cells found in vascular system.

The ingrowth of ECs from the existing vasculature at the site of anastomosis is one of the major sources of ECs that facilitate endothelialization of vascular grafts [28]. However, the ingrowth of vascular smooth muscle cells (VSMCs), known as anastomotic intimal hyperplasia, can also occur along with the ingrowth of ECs causing prosthetic graft failure [29]. Therefore, it is important that the luminal surface of a synthetic vascular prosthesis has a preferential binding ability towards EC, especially over VSMCs. As a consequence, in the present study, the ECE-1 expression and the

4

synthetic bigET-1 processing ability of EC was examined in comparison to those of vascular smooth muscle cell (VSMC), another major cell type of vasculature. Furthermore, using synthetic bigET-1-immobilized glass substrates and a rheology testing device, binding of ECs under hemodynamic shear stress was examined and compared to that of VSMCs.

2. Material and Methods

2.1 Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Allendale, NJ, USA), and human coronary artery smooth muscle cells (HCASMC) were purchased from ECACC (European collection of cell cultures, UK). HUVECs were maintained in endothelial growth medium (EGM2, Lonza) and HCASMCs were maintained in smooth muscle cell growth medium (SmGM2, Lonza). All cells were maintained at 37 °C in a 5% v/v CO_2 incubation chamber.

2.2 Detection of the ECE-1 expression in vascular cells

For the detection of ECE-1 expression in HCASMCs and HUVECs, cells (10^3cells/well) grown in a 4 well chamber slides (30104, SPL Life Sciences Col, Ltd, Pocheon City, Korea) were subjected to immunostaining using polyclonal goat IgG human ECE-1 primary antibodies (AF1784, R&S Systems, Minneapolis, MN, USA) and FITC-conjugated anti-goat secondary antibodies (705-096-147, Jackson ImmunoResearch Europe Ltd., Suffolk, UK). Briefly, the cells were washed with PBS and fixed in 4% w/v paraformaldehyde for 10 minutes at room temperature. After PBS wash, the cells were permeabilized in 0.1% v/v Triton X-100 for 5 minutes and then phosphate buffered saline (PBS) washed. After 30 minutes of blocking in 1% w/v bovine serum albumin in PBS, the cells were incubated with the primary antibodies (1:100, 2% w/v bovine serum albumin (BSA) in PBS) overnight at 4 °C. The cells were washed in PBS 3 times and successively incubated with the secondary antibodies (1:200, 2% w/v BSA in PBS) for additional 1 hour at room temperature. For visualizing the

cytoskeletal structure, F-actin was stained with Texas Red-X phalloidin (Invitrogen, Waltham, MA, USA) and the nuclei were stained with DAPI. Immunofluorescence was detected by confocal microscopy (LSM710; Carl Zeiss Microscopy GmbH, Jena, Germany).

2.3 Reverse transcription polymerase chain reaction (RT-PCR)

Briefly, total RNAs from HCASMCs and HUVECs were extracted using TRIzol reagent (Ambion, Waltham, MA, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 500 ng of RNA by AMV reverse transcriptase (Promega, Fitchburg, WI, USA). The primer sequences used were as follows: human ECE-1 (458bp): Forward: 5′-TGT CTT CCT CGC TGG AAG TT-3', Reverse: 5′-GCC GGA AAC ACA ATC TCA TT-3′; human β-actin (352bp): Forward: 5′-AAA CTG GAA CGG TGA AGG TG-3', Reverse: 5′-CTC AAG TTG GGG GAC AAA AA-3.

2.4 Enzymatic processing of bigET-1 by ECs in suspensions

Synthetic bigET-1 (25 ng, 023-10, Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) was incubated with various concentrations of suspended ECs ($0.25 \sim 8 \times 10^4$ cells/ml) in endothelial basal medium (EBM, Lonza) at 37°C, and 100μl of the supernatants were collected at 1 and 2 hours. The collected supernatants were centrifuged at 3,000 g for 5 minutes to precipitate the cells and the supernatants were used to detect the amount of ET-1 produced by enzymatic cleavage using Endothelin-1 Quantikine ELISA Kit (DET100, R&D systems, Minneapolis, MN, USA) according to the manufacturer recommended protocol. For the detection of bigET-1 cleavage in blood, 900μl of pooled blood collected from male balb/c athymic nude mice (5 weeks old, Koatech, Pyeoungtaek, Korea) via orbital blood collection was mixed with 100μl of EGM2 (Lonza) containing bigET-1 (0.1 μg/ml) and HUVECs (10~100 cells/ml) for 2 hours. The amount of ET-1 produced was determined by using human Endothelin-1 QuantiGlo ELISA Kit (QET00B, R&D systems). All experimental

6

procedures for animal studies were approved by the Committee for the Care and Use of Laboratory Animals of Catholic Kwandong University College of Medicine and were performed in accordance with the Committee's Guidelines and Regulations for Animal Care (CKU 01-2017-008).

2.5 Immobilization of bigET-1

Plain cover glass for the rheology testing chamber with diameter of 2.5 cm were cleaned with piranha solution (3/1, v/v, conc. H_2SO_4/30% v/v H_2O_2.) at 75 °C for 1 hour and rinsed thoroughly with MilliQ water. These substrates were immersed into a 1 wt.% toluene solution of 5-hexenyltrichlorosilane for 1 hour to obtain substrates having vinyl-terminated self-assembled monolayers (SAMs) [30]. The vinyl-functional groups were then oxidized to –COOH groups under Lemieux condition (0.5mM of $KMnO_4$, 14.7 mM of $NaIO_4$ and 3 mM of K_2CO_3 for 24 h), followed by washing with MilliQ water [31]. The formed carboxylic acid groups were further activated by using 45 mM 1-(3-(dimethylamino) propyl)-3-ethylcarbodiimide hydrochloride (EDC) and 15mM N-hydroxysuccinimide (NHS) for 3 hours at room temperature [32]. The glass substrates were then thoroughly rinsed with MilliQ water (Supplementary Figure 1A). To form amide bond between the activated carboxylic acid group of the glass substrates and the amine group of proteins, known concentration of protein solution was applied to the activated surface for up to 24 hours. Unbound proteins were washed off under running water for 5 minutes. Protein binding was verified by staining with Bradford solution after protein immobilization (Supplementary Figure 1B).

2.6 Enzymatic processing of surface-immobilized bigET-1 by vascular cells under static condition

Cover glass with activated carboxylic acid group was immersed and incubated in bigET-1 solution (10 ng/ml, 2 ml) overnight. The synthetic bigET-1-immobilized cover glass was placed in a 35mm culture dish, and either HUVECs ($4x10^4$ cells/ml, 2 ml) or HCASMCs ($8x10^4$ cells/ml, 2 ml) were seeded on top of the cover glass. The cells were cultured in a CO_2 incubation chamber for 24

hours under static condition. The culture media were collected and the amount of ET-1 produced was determined by using Endothelin-1 Quantikine ELISA Kit (R&D systems).

2.7 Vascular cell binding to surface-immobilized bigET-1 under shear stress

The test of cell binding under shear stress was performed using the method described previously with minor modifications [18]. On the day of the experiment, synthetic bigET-1-immobilized sample glass disks (immersed in 5 ml of 100 ng/ml bigET-1 solution for 3 hours) were thoroughly washed with MilliQ water and then placed in the sample chambers of the rheology testing device (Rheobasic®, Engtech GmbH, Ulm, Germany). Suspensions of HUVECs or HCASMCs (2×10^5 cells in 700 µl of the respective culture media) were applied to the respective sample chambers. Immediately, the cells were exposed to a shear stress of 5 dyne/cm^2 for 1 hour. After 1 hour of shearing, the sample disks were washed by 3 changes of culture medium, and microphotographs were taken from 8 different spots from each sample disk. To evaluate the cell type specific binding of the surface, the number of cells was counted from each high powered field (100xHPF) microphotograph.

2.8 Comparison of bigET-1 to other adhesion proteins for EC binding under shear stress

To verify the EC-specificity of the synthetic bigET-1-immobilized surface, the adhesion of HUVECs and HCASMCs to the BSA-immobilized surface and the human collagen type IV-immobilized surface under shear stress was examined. For BSA-immobilized surface, activated sample glass disks were immersed in a BSA solution (10 mg/ml, 5 ml) for 3 hours. For collagen-immobilized surface, the sample glass disks were immersed in a collagen solution (0.1 mg/ml, 5 ml) for 3 hours. Suspensions of HUVECs or HCASMCs (2×10^5 cells in 700 µl of the respective culture media) were applied to the respective sample chambers, and the cells were exposed to a shear stress of 5 dyne/cm^2 for 30 minutes.

2.9 Statistical analysis

For all quantification analyses, at least three independent experiments were performed. The quantitative data were expressed as the arithmetic means ± S.D. (standard deviation) of at least 3 independent experiments. For two sample comparisons, a two-sided *t*-test was used. *P* values less than 0.05 were considered to be significant.

3. Results

3.1 ECE-1 expressions in vascular cells

To examine the expression of ECE-1 in major vascular cells, HUVECs and HCASMCs were subjected to immunocytochemical staining using anti-human specific ECE-1 antibodies. The expression of ECE-1 was apparently pronounced in HUVECs compared to that of HCASMCs (Fig. 1A). The mRNA expressions of ECE-1 in HUVECs and HCASMCs were examined by RT-PCR, and the ECE-1 mRNA expression in HUVEC was significantly higher compared to that of HCASMC (2.35 ± 0.31 vs. 0.3 9± 0.02, arbitrary unit, p<0.001) (Fig. 1B).
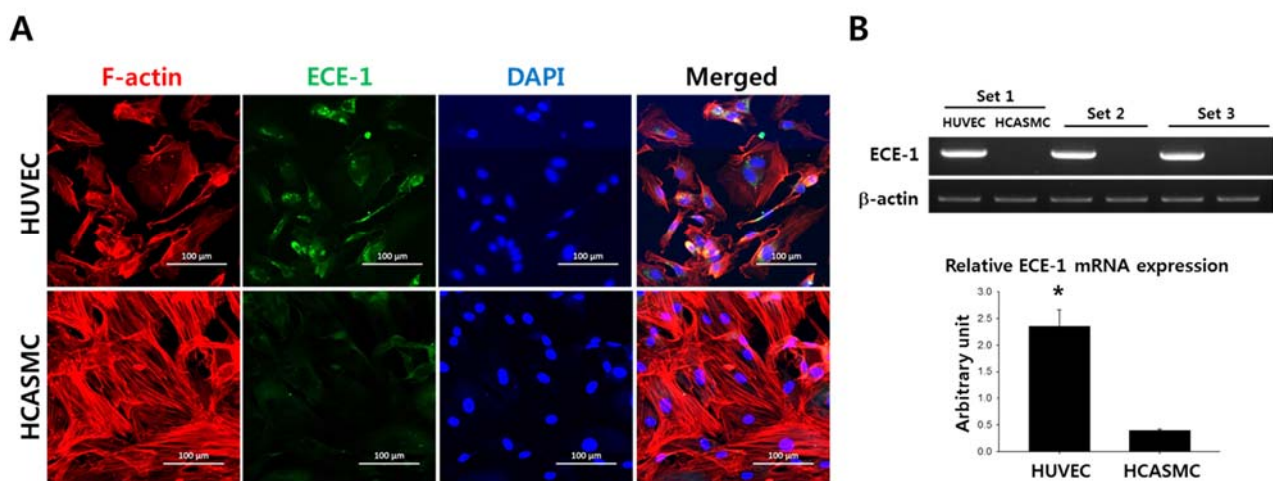

Figure 1. Expression of ECE-1 in ECs and VSMCs. (A) Representative immuno-cytochemical staining images of ECE-1 in HUVECS and HCASMCs. The cells were seeded in a 4 well chamber slide (10^3 cells/well) and allowed to attach and spread for 24 hours. The expression of human ECE-1 was immunostained using human ECE-1-specific primary antibodies and FITC-conjugated secondary antibodies. Cytoskeletal organization and the nuclei were visualized by Texas Red-X phalloidin and

DAPI staining, respectively. Scale bar=100μm. (B) The mRNA expression of ECE-1 in HUVECs and HCASMCs was determined by RT-PCR. The quantitative data were expressed as the means ± S.D. of at least 3 independent experiments. *p<0.001.

3.2 EC number-dependent enzymatic processing of synthetic bigET-1 in suspension

To examine whether artificially synthesized bigET-1 could be processed by ECs, 25 ng of synthesized bigET-1 was incubated with increasing number of HUVECs ($0-8\times10^4$ cells/ml) in culture media for up to 2 hours. As shown in the Figure 2A, the amount of ET-1 produced significantly increased with EC numbers in samples collected at 1 hour and 2 hour (*p<0.05, significance indications for 2 hour samples are not shown). Furthermore, the amount of ET-1 produced by 2 hours of incubation was significantly higher compared to that by 1 hour incubation at all the cell numbers tested (#p<0.01). Even smaller number of HUVECs (80-100 cells/ml) suspended in mouse blood were able to significantly increase the amount of ET-1 produced by the enzymatic cleavage of synthetic bigET-1 (100ng/ml) within 2 hours (Fig. 2B).
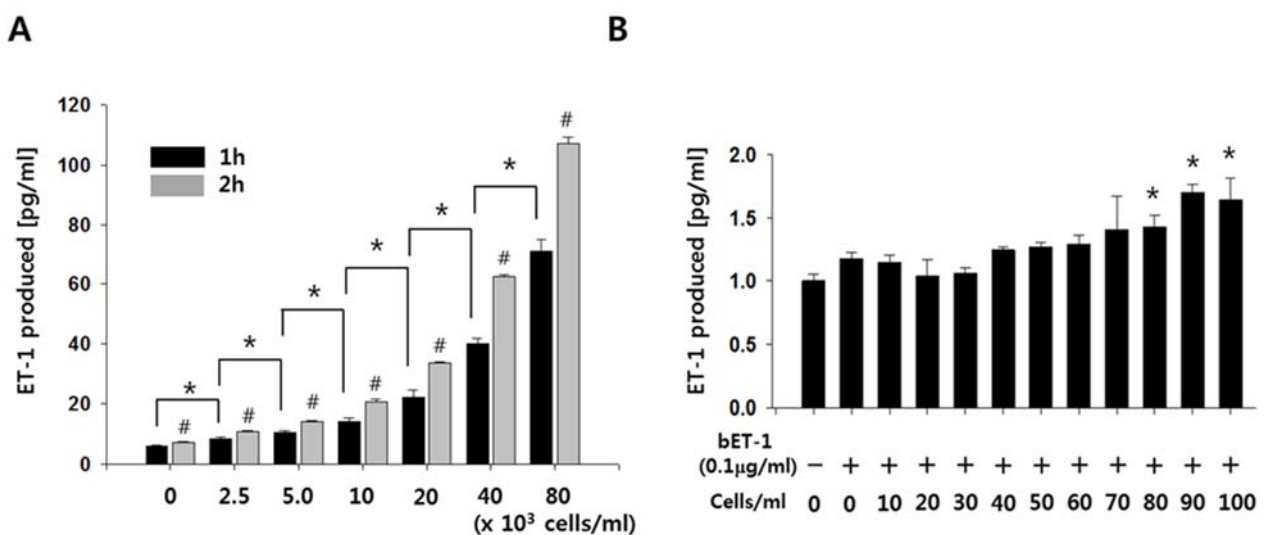

Figure 2. EC number-dependent cleavage of synthetic bigET-1. (A) Synthesized bigET-1 (25 ng) was incubated with increasing number of ECs at 37 °C for up to 2 hours, and the amount of ET-1 produced by the cleavage of bigET-1 was measured using an ET-1 ELISA kit. *p<0.05, significance indications for 2 hour samples are not shown. #p<0.01 compared to matching 1 hour sample. (B) For the detection of synthetic bigET-1 cleavage in blood, 900μl of mouse blood was mixed with 100μl of endothelial growth medium containing synthetic bigET-1 (100ng/ml) and HUVECs (0~100 cells/ml)

for 2 hours. The amount of ET-1 produced was determined by using an ET-1 ELISA kit. The quantitative data were expressed as the means ± S.D. of at least 3 independent experiments. *p<0.05.

3.3 Enzymatic processing of surface-immobilized synthetic bigET-1 under static condition

To investigate any possible adverse effect of surface modification using synthetic bigET-1 on cell adhesion and also to verify that the surface-immobilized synthetic bigET-1 could be processed in an EC-specific manner, HUVECs (4×10^4 cells/ml) or HCASMCs (8×10^4 cells/ml) were seeded on top of the bigET-1-immobilized cover glass. Upon microscopic examination, no unusual abnormality in either initial cell adhesion (10 minutes after seeding) or subsequent cell spreading (24 hours after seeding) was observed. Furthermore, under static culture condition, no apparent EC-specific binding and spreading effect was observed (Fig. 3A). However, HUVECs processed significantly larger amount of the surface immobilized synthetic bigET-1 compared to HCASMCs so that the amount of ET-1 produced by HUVECs was approximately 10-times larger than that produced by HCASMCs (102.86 ± 5.13 vs. 9.75 ± 0.74 pg/ml, p<0.001) (Fig. 3B). Considering the number of HCASMCs seeded was 2-times higher than that of HUVECs, actual fold difference between HUVEC and HCASMC could have been close to 20.
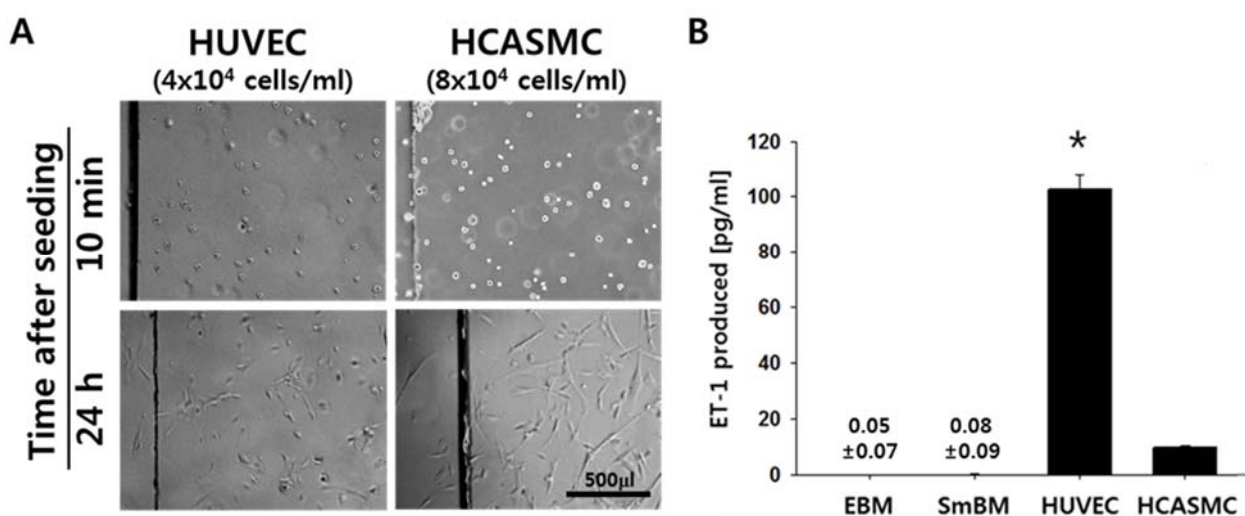

11

Figure 3. Enzymatic cleavage of immobilized bigET-1 by HUVEC and HCASMC. Either HUVECs (4×10^4cells/ml, 2ml) or HCASMCs (8×10^4cells/ml, 2ml) were cultured on bigET-1-immobilized glass cover slides for 24 hours. (A) Representative images of vascular cells adhere (10 minutes after seeding) and spread (24 hours after seeding) on the bigET-1-immobilized surface. (B) The amount of ET-1 produced by enzymatic cleavage of bigET-1 was measured at 24 hours using an ET-1 Elisa kit. EBM: endothelial basal medium, SmBM: smooth muscle basal medium. The quantitative data were expressed as the means ± S.D. of at least 3 independent experiments. *p<0.001 compared to HCASMC.

3.4 EC-preferential binding to synthetic bigET-1- immobilized surface under shear stress

Once implanted, vascular prosthesis will be continuously exposed to a physiologic shear stress caused by blood flow. Therefore, an ideal blood contacting surface should be able to preferentially attract ECs under shear stress. To assess the EC-specific binding capacity of the synthetic bigET-1-immobilized surface, adherence of HUVECs and HCASMCs (2×10^5 cells in 700 µl of the respective culture media) to the synthetic bigET-1-immobilized surface was evaluated under shear stress. The number of HUVECs adhered to the surface after 1 hour of shear stress (5 dyne/cm^2) was significantly higher compared to that of HCASMCs (46.25 ± 12.61 vs. 15.00 ± 3.69 cells/100xHPF, p<0.001) (Fig. 4A). Additionally, adherence of HUVECs and HCASMCs to BSA-immobilized or collagen type IV-immobilized surface under shear stress was also examined, and EC-specific affinity of the modified surface was only observed in the synthetic bigET-1-immobilized surfaces (Fig. 4B). Specifically, both the BSA + bigET-1-immobilized surface (28.75 ± 12.45 vs. 0.50 ± 0.87 cells/100xHPF, p<0.005) and the bigET-1-immobilized surface (18.00 ± 3.74 vs. 1.25 ± 1.30 cells/100xHPF, p<0.001) showed statistically significant EC-preferential binding over VSMCs. Although the difference between the number of HUVECs and the number of HCASMCs adhered to the BSA-immobilized surface was also statistically significant (3.50 ± 1.00 vs. 0.00 ± 0.00 cells/100xHPF, p<0.001), the cell numbers adhered was too low to have scientific significance.
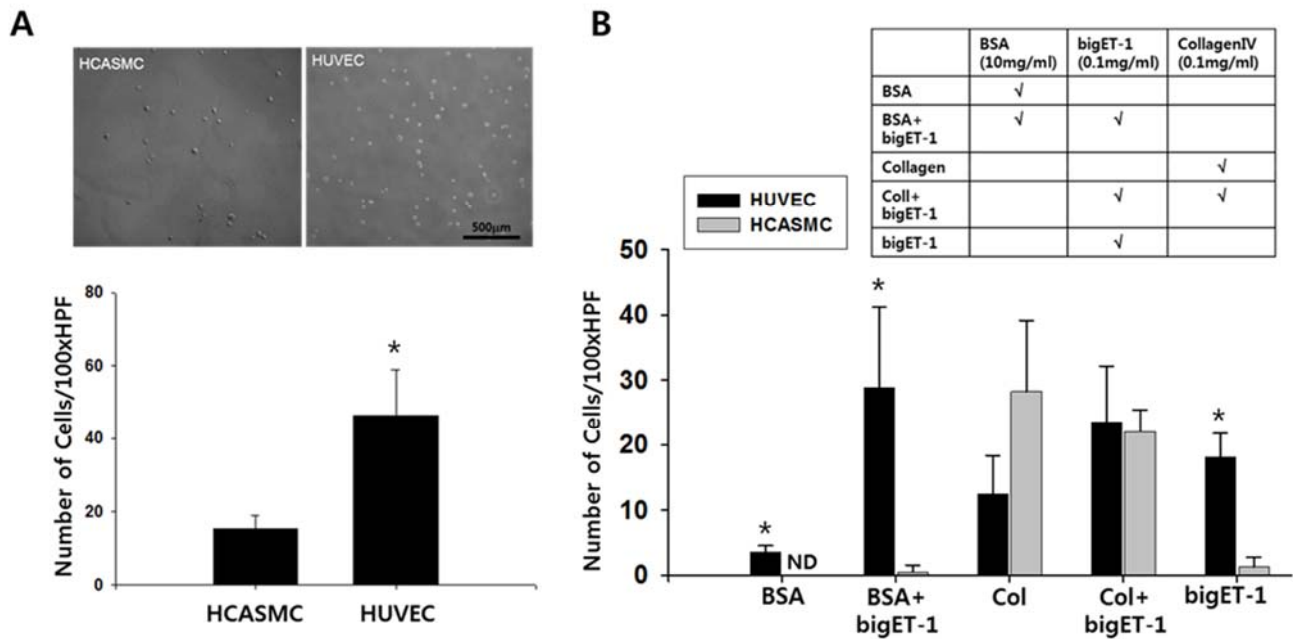
12

Figure 4. EC-specific binding of bigET-1-immobilized surface under shear stress. (A) Suspension of HUVECs or HCASMCs (2×10^5 cells) were allowed to bind to the bigET-1-immobilized surface (immersed in 5ml of 100ng/ml bigET-1 solution for 3 hours) under shear stress (5dyne/cm^2, 1 hour). After 1 hour of shearing, the samples were washed by 3 changes of culture medium, and high powered field microphotographs (100x HPF) were taken from 8 different spots from each sample disk. The number of cells was counted from each microphotograph. (B) Glass surface was modified with BSA, Collagen, bigET-1, or combinations of them as indicated. Proteins allowed to form amide bonds to the surface for 3 hours. HUVECs and HCASMCs (2×10^5 cells) were allowed to bind under shear stress (5dyne/cm^2) for 30minutes. The number of surface-bound cells was counted from each 100x HPF microphotograph. ND: not detected. The quantitative data were expressed as the means ± S.D. of at least 3 independent experiments. Statistical significance compared to the matching HCASMC group is indicated.

4. Discussion

For cardiovascular implants, especially for small diameter vascular prostheses, a fully hemocompatible blood contacting surface is important in order to prevent current vascular implant-associated problems such as implant obstruction due to the formation of thrombus or intimal hyperplasia that can lead to the failure of the implant [33, 34]. Currently, there is no single surface modification strategy commonly accepted as the best approach to create a surface with EC-specific binding capability [35]. Therefore, testing various options to engineer hemocompatible surfaces is well

justified. In the present study, a substrate-enzyme affinity (SEA)-based surface modification was tested as a strategy to give an artificial surface EC-specific binding capability. In our approach a cell type-specific enzyme shall react with the corresponding substrates immobilized on a surface, thus enabling a cell type-specific adhesion under shear stress to resist an detachment of EC by shear forces.

The major sources of ECs for the endothelialization of implanted vascular grafts include the ingrowth of ECs from the existing vasculature at the site of anastomosis [28]. However, existing VSMCs can also migrate into the implanted vascular grafts causing prosthetic graft failure [29]. Therefore, it was important to choose an EC plasma membrane enzyme that is preferentially expressed in ECs over VSMCs. The EC plasma membrane enzyme ECE-1 has been reported to be mostly expressed in ECs of blood vessels [36, 37], and our data also confirmed the pronounced expression of ECE-1 in ECs compared to VSMCs (Fig. 1). The major substrate of ECE-1 is bigET-1, which is enzymatically cleaved by ECE-1 to produce bioactive ET-1 [38]. Although big ET-1 has two additional isoforms, bigET-2 and bigET-3, the specificity of ECE-1 for bigET-1 is higher compared to the others [39]. Furthermore, a previous study reported that VSMCs could also process bigET-1, but the enzyme activity of VSMCs was negligible compared to that of ECs [40]. Consequently, bigET-1 was selected as a potential candidate molecule for the surface modification.

In the enzyme-substrate interaction, conformational stability of the participating proteins (i.e., both enzymes and substrates) is critical for enzymatic activity [41-43]. In other words, 3D structures of both the enzyme and the corresponding substrate may significantly affect the enzymatic activity. Unlike endogenous bigET-1 that has 2 disulfide bonds (Cys^1-Cys^{15} and Cys^3-Cys^{11}) known to significantly affect the enzyme activity of ECE-1 [44, 45], synthesized bigET-1 used in the present study was in a linear form without any disulfide bonds. Therefore, EC-dependent enzymatic processing of the synthetic bigET-1 was then examined prior to surface immobilization or shear stress test. As shown in the Figure 2A, the amount of ET-1 produced was proportional to the number of ECs,

14

indicating that the synthesized bigET-1 could be properly processed by ECs even without disulfide bonds.

Besides aforementioned migrating ECs at the site of anastomosis, blood circulating ECs and/or endothelial progenitor cells (EPCs) could be another major source of ECs for the endothelialization of implanted vascular prostheses. According to a previous study, the expression of ECE-1 in EPC-derived progenitor cells from both cord blood and G-CSF mobilized peripheral blood was higher than in HUVECs [46]. Furthermore, implanted vascular prostheses will be continuously exposed to blood flow. Therefore, it was critical to confirm that the enzyme-substrate interaction we designed works properly in the presence of blood. The data using mouse blood indicated that even smaller number of HUVECs successfully produced ET-1 from synthetic bigET-1 in blood (Fig. 2B). Speaking of properly function in blood, ECE-1 is a member of the M13 subfamily of zinc endopeptidases which also includes the Kell blood group protein, a blood-borne enzyme [47]. Since they share some sequence homology and structural similarities [48], there was a concern that the Kell blood group protein also interacts with bigET-1. However, literature search indicated that the Kell blood group protein preferentially processes bigET-3 not bigET-1 [49]. Therefore, it was assumed that other ECE-1 related enzymes such as the Kell blood group protein, or the cells expressing such enzymes, less likely interact with the bigET-1 until empirically proven otherwise in future studies. After taking into account all these considerations, ECE-1 and bigET-1 pair was finally selected as the model system to be tested in the present study.

For the present study, plain glass was used as the base-substrate because it can be easily functionalized with various chemical functional groups such as carboxylic acid, amine, and aldehyde. Additionally, transparent glass substrate enables the observation of adhered cells under the microscope without extra cell staining steps. The chemical modification starts with the formation of carboxylic group. The advantage of this modification is that the surface carboxylic groups can be easily activated and reacted with free amine groups of proteins to form an amide bond, which is facilitated in aqueous media by the coupling reagents EDC and NHS (Supplementary Fig.1A). Using this strategy, one can

chemically immobilize various biomolecules containing free amine groups such as amine-modified DNA, peptides, and proteins [50]. A Bradford-staining showed that the modified surface successfully bound common protein BSA (Supplementary Fig.1B). Another important advantage of this strategy is that it is also easily applicable to various polymer films that could potentially serve as base material for vascular grafts [51].

On the other hand, one of the possible disadvantages of this approach is that the orientation of binding cannot be controlled. The binding strategy used was based on the random covalent bonding between the activated carboxyl groups of the surface and the free amine groups of applied proteins. Usually, there are more-than-one free amine groups presented on proteins, and which of them participates in the amine bonds cannot be controlled. Furthermore, ECE-1 recognizes the sequence of bigET-1 from histone 27 to glycine 34 (His-Val-Val-Pro-Tyr-Gly-Leu-Gly) to physically interact [52]. Therefore, it could be possible that if the free amine groups of the histone 27 or the proline 30 were used up for the immobilization, ECE-1 might not be able to recognize the synthetic bigET-1 immobilized. To investigate such possibility, ECs and VSMCs were cultured on a synthetic bigET-1-immobilized surface for 24 hours under static condition. Another important purpose of the static culture on the synthetic bigET-1-immobilized surface was to examine whether the synthetic bigET-1-immobilized surface supports proper adhesion and subsequent spreading of the cells.

According to our data, both HUVECs and HCASMCs properly attached and spread on the synthetic bigET-1-immobilized surface (Fig. 3A), indicating that there was no significant adverse effect of surface modification on cell adhesion. Furthermore, more importantly, even less number of HUVECs produced significantly larger amount of ET-1 compared to HCASMCs (Fig. 3B), strongly suggesting that at least some of the synthetic bigET-1 immobilized to the surface maintained ECE-1 recognition motif available. Nevertheless, exactly how much of the immobilized synthetic bigET-1 maintained ECE-1 recognition motif available was not determined, and it remains one of the limitations of the present study. To eliminate the uncertainty of recognition motif availability after

16

immobilization, different immobilization strategies that can give more control over the binding orientation such as the His-tag approach [53] will be considered in future studies.

Since the SEA-based surface modification strategy is intended to work with blood flow, EC-specific binding capacity of the synthetic bigET-1-immobilized surface under shear stress was examined. Compared to HCASMCs, significantly higher number of HUVECs adhered to the synthetic bigET-1-immobilized surface under shear stress (Fig. 4A). This result highlights an important aspect of the SEA-based surface modification strategy that the enzyme-substrate interaction was specific to ECs and strong enough to withstand the applied shear stress of 5 dyne/cm^2, a shear stress that occurs in many vascular regions [54]. Furthermore, to exclude the possibility that the observed EC-specific binding under shear stress was due to general protein modification rather than due to the synthetic bigET-1 modification, additional rheology testing using BSA-immobilized and collagen-immobilized samples were conducted.

According to our data, EC-specific affinity of the modified surface was observed only when the synthetic bigET-1 was used (both alone and in combination with BSA) for the surface modification (Fig. 4B). Although the BSA-immobilized surface also showed significant difference between the number of HUVECs and the number of HCASMCs adhered, even the number of HUVECs adhered was insignificant. Taken together, these results confirmed that the modification with synthetic bigET-1 could give a surface EC-specific binding capacity under shear stress. In small diameter vascular prostheses, that are intended to replace or bypass the coronary arteries, the arterial wall shear stress ranges from 7 to 70 dynes/cm^2 [55, 56]. Therefore, one can argue that withstanding a shear stress of 5 dyne/cm^2 may not be clinically significant as it is. Nevertheless, as a proof of concept study, testing the feasibility of using SEA-based modification to engineer a surface selectively attracting ECs under shear stress has its own significance.

Another limitation of the present study is the lack of proven correlation between the amount of the protein immobilized to the surface and the number of cells adhered under shear stress. It would be

17

necessary to know the protein density on the surface after the modification in order to optimize this SEA-based approach. In the future, a more thorough surface analysis following the surface modification such as infrared spectroscopy or Raman spectroscopy [57] and/or multicomponent protein patterning in combination with other proteins that support EC binding and proliferation [58] would be helpful to improve the surface modification strategy. Since enzymatically cleaved bigET-1 produces the most potent vasoconstrictor ET-1 [59], future studies should use bigET-1-derived peptides, not the original bigET-1 itself. Using such derivatives will enable us to achieve EC-specific binding without causing the problem of excessive ET-1 production.

In the present study, the SEA-based approach was explored with regard to its potential as a cell type-specific surface modification strategy. Our data indicated that this SEA-based approach worked properly to give a surface EC-specific binding capacity. Even the most recent EPC capturing study still utilized a more than decade-old strategy based on the interaction between EC expressed molecules and its corresponding antibodies [60, 61]. Therefore, the SEA-based strategy could be the next generation alternative to engineer a shear-resistant EC-specific binding surface for hemocompatible cardiovascular implants.

Acknowledgements

References

[1]   Butler D. UN targets top killers. Nature. 2011;477(7364):260-1.

[2]   Ramallal R, Toledo E, Martinez-Gonzalez MA, Hernandez-Hernandez A, Garcia-Arellano A, Shivappa N, et al. Dietary Inflammatory Index and Incidence of Cardiovascular Disease in the SUN Cohort. PLoS One. 2015;10(9).

[3]   Anadol R, Dimitriadis Z, Polimeni A, Wendling F, Gonner S, Ullrich H, et al. Bioresorbable everolimus-eluting vascular scaffold for patients presenting with non STelevation-acute coronary syndrome: A three-years follow-up1. Clin Hemorheol Microcirc. 2018;69(1-2):3-8.

[4]   Locker C, Schaff HV, Dearani JA, Daly RC. Improved late survival with arterial revascularization. Ann Cardiothorac Surg. 2013;2(4):467-74.

[5]   Zilla P, Bezuidenhout D, Human P. Prosthetic vascular grafts: wrong models, wrong questions and no healing. Biomaterials. 2007;28(34):5009-27.

[6]   Anderson DEJ, Glynn JJ, Song HK, Hinds MT. Engineering an Endothelialized Vascular Graft: A Rational Approach to Study Design in a Non-Human Primate Model. PLoS One. 2014;9(12):e115163.

[7]   Paternotte E, Kerdjoudj H, Kokten T, Stoltz J-F, Kearney-Schwartz A, Voegel J-C, et al. Endothelialized and preconditioned natural umbilical arteries with long term patency open the route for future human uses. Clin Hemorheol Microcirc. 2013;54(3):223-34.

[8]   Jung F, Wischke C, Lendlein A. Degradable, Multifunctional Cardiovascular Implants: Challenges and Hurdles. MRS Bull. 2010;35(8):607-13.

[9]   Mayer A, Roch T, Kratz K, Lendlein A, Jung F. Pro-angiogenic CD14++ CD16+ CD163+ monocytes accelerate the in vitro endothelialization of soft hydrophobic poly(n-butyl acrylate) networks. Acta Biomater. 2012;8(12):4253-9.

[10] Krüger A, Fuhrmann R, Jung F, Franke RP. Influence of the coating with extracellular matrix and the number of cell passages on the endothelialization of a polystyrene surface. Clin Hemorheol Microcirc. 2015;60(1):153-61.

[11] Goh ET, Wong E, Farhatnia Y, Tan A, Seifalian AM. Accelerating in situ endothelialisation of cardiovascular bypass grafts. Int J Mol Sci. 2015;16(1):597-627.

[12] Schulz C, von Ruesten-Lange M, Krueger A, Lendlein A, Jung F. Adherence and shear-resistance of primary human endothelial cells on smooth poly(ether imide) films. Clin Hemorheol Microcirc. 2014;57(2):147-58.

[13] Neufurth M, Wang X, Tolba E, Dorweiler B, Schroeder HC, Link T, et al. Modular Small Diameter Vascular Grafts with Bioactive Functionalities. PLoS One. 2015;10(7):e0133632.

[14] Deutsch M, Meinhart J, Fischlein T, Preiss P, Zilla P. Clinical autologous in vitro endothelialization of infrainguinal ePTFE grafts in 100 patients: a 9-year experience. Surgery. 1999;126(5):847-55.

[15] Dohmen PM, Pruss A, Koch C, Borges AC, Konertz W. Six years of clinical follow-up with endothelial cell-seeded small-diameter vascular grafts during coronary bypass surgery. Journal of tissue engineering. 2013;4:Article ID 2041731413504777.

[16] Ji Q, Zhang S, Zhang J, Wang Z, Wang J, Cui Y, et al. Dual Functionalization of Poly(epsilon-caprolactone) Film Surface through Supramolecular Assembly with the Aim of Promoting In Situ Endothelial Progenitor Cell Attachment on Vascular Grafts. Biomacromolecules. 2013;14(11):4099-107.

[17] Hoepken S, Fuhrmann R, Jung F, Franke RP. Shear resistance of human umbilical endothelial cells on different materials covered with or without extracellular matrix: Controlled in-vitro study. Clin Hemorheol Microcirc. 2009;43(1):157-66.

[18] Franke RP, Grafe M, Schnittler H, Seiffge D, Mittermayer C, Drenckhahn D. Induction of human vascular endothelial stress fibres by fluid shear stress. Nature. 1984;307(5952):648-9.

[19] Furth ME, Atala A, Van Dyke ME. Smart biomaterials design for tissue engineering and regenerative medicine. Biomaterials. 2007;28(34):5068-73.

[20] Lange M, Braune S, Luetzow K, Richau K, Scharnagl N, Weinhart M, et al. Surface functionalization of poly(ether imide) membranes with linear, methylated oligoglycerols for reducing thrombogenicity. Macromol Rapid Commun. 2012;33(17):1487-92.

[21] Ullm S, Krüger A, Tondera C, Gebauer TP, Neffe AT, Lendlein A, et al. Biocompatibility and inflammatory response in vitro and in vivo to gelatin-based biomaterials with tailorable elastic properties. Biomaterials. 2014;35(37):9755-66.

[22] Ruoslahti E. RGD and other recognition sequences for integrins. Annu Rev Cell Dev Biol. 1996;12:697-715.

[23] Hersel U, Dahmen C, Kessler H. RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. Biomaterials. 2003;24(24):4385-415.

[24] Schnittler HJ, Franke RP, Akbay U, Mrowietz C, Drenckhahn D. Improved in vitro rheological system for studying the effect of fluid shear stress on cultured cells. American Journal of Physiology - Cell Physiology. 1993;265(1):C289-C98.

[25] Hamada Y, Nokihara K, Okazaki M, Fujitani W, Matsumoto T, Matsuo M, et al. Angiogenic activity of osteopontin-derived peptide SVVYGLR. Biochem Biophys Res Commun. 2003;310(1):153-7.

[26] Plouffe BD, Njoka DN, Harris J, Liao J, Horick NK, Radisic M, et al. Peptide-Mediated Selective Adhesion of Smooth Muscle and Endothelial Cells in Microfluidic Shear Flow. Langmuir. 2007;23(9):5050-5.

[27] Koshland DE. Application of a Theory of Enzyme Specificity to Protein Synthesis. Proc Natl Acad Sci U S A. 1958;44(2):98-104.

[28] Tsuchida H, Wilson SE, Ishimaru S. Healing mechanisms of high-porosity PTFE grafts: significance of transmural structure. J Surg Res. 1997;71(2):187-95.
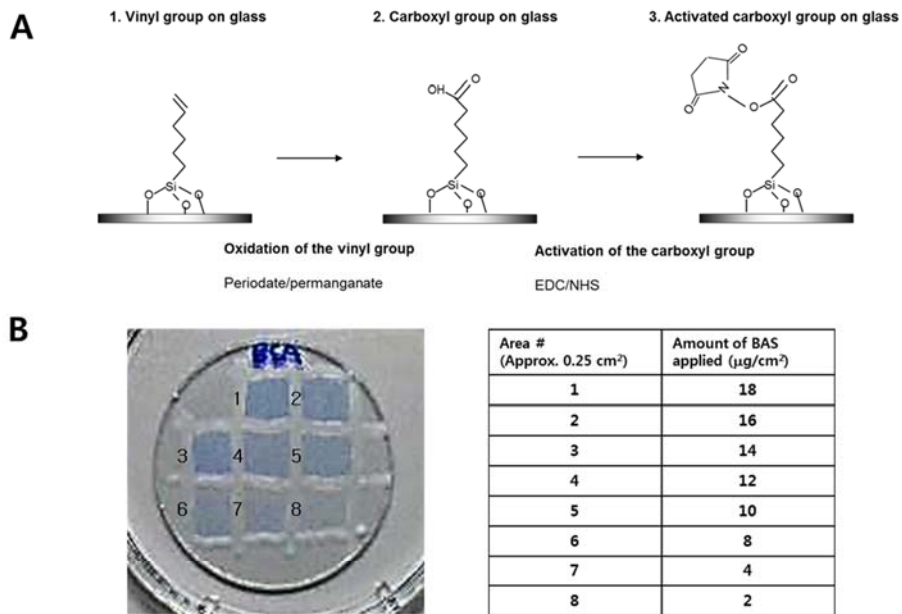
21

[29] Clowes AW, Gown AM, Hanson SR, Reidy MA. Mechanisms of arterial graft failure. 1. Role of cellular proliferation in early healing of PTFE prostheses. Am J Pathol. 1985;118(1):43-54.

[30] Park TJ, Lee K-B, Lee SJ, Park JP, Lee Z-W, Lee SY, et al. Micropatterns of Spores Displaying Heterologous Proteins. J Am Chem Soc. 2004;126(34):10512-3.

[31] Wasserman SR, Tao YT, Whitesides GM. Structure and Reactivity of Alkylsiloxane Monolayers Formed by Reaction of Alkyltrichlorosilanes on Silicon Substrates. Langmuir. 1989;5(4):1074-87.

[32] Ganesan R, Yoo SY, Choi JH, Lee SY, Kim JB. Simple micropatterning of biomolecules on a diazoketo-functionalized photoresist. J Mater Chem. 2008;18(6):703-9.

[33] Gorbet MB, Sefton MV. Biomaterial-associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes. Biomaterials. 2004;25(26):5681-703.

[34] Braune S, Gross M, Walter M, Zhou S, Dietze S, Rutschow S, et al. Adhesion and activation of platelets from subjects with coronary artery disease and apparently healthy individuals on biomaterials. J Biomed Mater Res B Appl Biomater. 2016;104(1):210-7.

[35] Reviakine I, Jung F, Braune S, Brash JL, Latour R, Gorbet M, et al. Stirred, shaken, or stagnant: What goes on at the blood-biomaterial interface. Blood Rev. 2017;31(1):11-21.

[36] Dawson LA, Maitland NJ, Berry P, Turner AJ, Usmani BA. Expression and Localization of Endothelin-Converting Enzyme-1 in Human Prostate Cancer. Exp Biol Med. 2006;231(6):1106-10.

[37] Takahashi M, Fukuda K, Shimada K, Barnes K, Turner AJ, Ikeda M, et al. Localization of Rat Endothelin-Converting Enzyme to Vascular Endothelial-Cells and Some Secretory-Cells. Biochem J. 1995;311:657-65.

[38] Houde M, Desbiens L, D'Orleans-Juste P. Endothelin-1: Biosynthesis, Signaling and Vasoreactivity. Adv Pharmacol. 2016;77:143-75.

[39] Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature. 1988;332(6163):411-5.

[40] Matsumura Y, Ikegawa R, Tsukahara Y, Takaoka M, Morimoto S. Conversion of big endothelin-1 to endothelin-1 by two types of metalloproteinases derived from porcine aortic endothelial cells. FEBS Lett. 1990;272(1–2):166-70.

[41] Csermely P, Palotai R, Nussinov R. Induced fit, conformational selection and independent dynamic segments: an extended view of binding events. Trends Biochem Sci. 2010;35(10):539-46.

[42] Kovermann M, Grundstrom C, Sauer-Eriksson AE, Sauer UH, Wolf-Watz M. Structural basis for ligand binding to an enzyme by a conformational selection pathway. Proc Natl Acad Sci U S A. 2017;114(24):6298-303.

[43] Thyparambil AA, Wei Y, Latour RA. Experimental characterization of adsorbed protein orientation, conformation, and bioactivity. Biointerphases. 2015;10(1):019002.

[44] Fahnoe DC, Johnson GD, Herman SB, Ahn K. Disulfide bonds in big ET-1 are essential for the specific cleavage at the Trp(21)-Val(22) bond by soluble endothelin converting enzyme-1 from baculovirus/insect cells. Arch Biochem Biophys. 2000;373(2):385-93.

[45] Darby N, Creighton TE. Disulfide bonds in protein folding and stability. Methods Mol Biol. 1995;40:219-52.

[46] Ott I, Keller U, Knoedler M, Götze KS, Doss K, Fischer P, et al. Endothelial-like cells expanded from CD34+ blood cells improve left ventricular function after experimental myocardial infarction. The FASEB Journal. 2005;19:992-4.

[47] Francis F, Hennig S, Korn B, Reinhardt R, Dejong P, Poustka A, et al. A Gene (Pex) with Homologies to Endopeptidases Is Mutated in Patients with X-Linked Hypophosphatemic Rickets. Nat Genet. 1995;11(2):130-6.

[48] Turner AJ, Tanzawa K. Mammalian membrane metallopeptidases: NEP, ECE, KELL, and PEX. The FASEB Journal. 1997;11(5):355-64.

[49] Lee S, Lin M, Mele A, Cao Y, Farmar J, Russo D, et al. Proteolytic Processing of Big Endothelin-3 by the Kell Blood Group Protein: Presented in part in abstract form at the 40th annual meeting of the American Society of Hematology, December, 1998. Blood. 1999;94(4):1440-50.

[50] Ganesan R, Lee H-J, Kim J-B. Photoactive Diazoketo-Functionalized Self-Assembled Monolayer for Biomolecular Patterning. Langmuir. 2009;25(16):8888-93.

[51] Choi J-H, Ganesan R, Kim D-K, Jung C-H, Hwang I-T, Nho Y-C, et al. Patterned Immobilization of Biomolecules by Using Ion Irradiation-Induced Graft Polymerization. Journal of Polymer Science Part A-Polymer Chemistry. 2009;47(22):6124-34.

[52] Okada K, Arai Y, Hata M, Matsuyama K, Yano M. Big endothelin-1 structure important for specific processing by endothelin-converting enzyme of bovine endothelial cells. Eur J Biochem. 1993;218(2):493-8.

[53] Nakaji-Hirabayashi T, Kato K, Arima Y, Iwata H. Oriented immobilization of epidermal growth factor onto culture substrates for the selective expansion of neural stem cells. Biomaterials. 2007;28(24):3517-29.

[54] Kruger-Genge A, Blocki A, Franke RP, Jung F. Vascular Endothelial Cell Biology: An Update. Int J Mol Sci. 2019;20(18).

[55] Papaioannou TG, Stefanadis C. Vascular wall shear stress: basic principles and methods. Hellenic J Cardiol. 2005;46(1):9-15.

[56] Tenekecioglu E, Torii R, Katagiri Y, Chichareon P, Asano T, Miyazaki Y, et al. Post-implantation shear stress assessment: an emerging tool for differentiation of bioresorbable scaffolds. Int J Cardiovasc Imaging. 2019;35(3):409-18.

[57] Staufenbiel S, Merino M, Li W, Huang MD, Baudis S, Lendlein A, et al. Surface characterization and protein interaction of a series of model poly[acrylonitrile-co-(N-vinyl pyrrolidone)] nanocarriers for drug targeting. Int J Pharm. 2015;485(1-2):87-96.

[58] Ganesan R, Kratz K, Lendlein A. Multicomponent protein patterning of material surfaces. J Mat Chem. 2010;20:7322-31.

[59] Agapitov AV, Haynes WG. Role of endothelin in cardiovascular disease. Journal of Renin-Angiotensin-Aldosterone System. 2002;3(1):1-15.

[60] Huang YH, Xu Q, Shen T, Li JK, Sheng JY, Shi HJ. Prevention of in-stent restenosis with endothelial progenitor cell (EPC) capture stent placement combined with regional EPC transplantation: An atherosclerotic rabbit model. Cardiol J. 2019;26(3):283-91.

[61] Garg S, Duckers HJ, Serruys PW. Endothelial progenitor cell capture stents: will this technology find its niche in contemporary practice? Eur Heart J. 2010;31(9):1032-5.

Supplementary Figure 1

A

1. Vinyl group on glass   2. Carboxyl group on glass   3. Activated carboxyl group on glass

Oxidation of the vinyl group   Activation of the carboxyl group

Periodate/permanganate   EDC/NHS

B

Area # (Approx. 0.25 cm^2)	Amount of BAS applied (μg/cm^2)
1	18
2	16
3	14
4	12
5	10
6	8
7	4
8	2

Schematics of protein immobilization and verification. (A) To immobilize proteins, glass substrate was first modified with vinyl-functional group. The vinyl-functional groups were then oxidized to –COOH groups and further activated to make amide bonds with the amine groups of proteins. (B) Samples stained with Bradford solution after immobilization of increasing concentrations of BSA. EDC: 1-(3-(dimethylamino) propyl)-3-ethylcarbodiimide hydrochloride, NHS: N-hydroxysuccinimide.