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Decorin Mimic Promotes Endothelial Cell Health in Endothelial Monolayers and Endothelial-Smooth Muscle Co-Cultures

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Abstract

Non-specific cytotoxins, including paclitaxel and sirolimus analogs, currently utilized as anti-restenotic therapeutics affect not only smooth muscle cells (SMC), but also neighboring vascular endothelial cells (EC). These drugs inhibit the formation of an intact endothelium following vessel injury and thus, emphasizing the critical need for new candidate therapeutics. Utilizing our *in vitro* models, including EC monolayers and both hyperplastic and quiescent EC-SMC co-cultures, we investigated the ability of DS-SILY₂₀, a decorin mimic, to promote EC health. DS-SILY₂₀ increased EC proliferation and migration by 1.5- and 2-fold, respectively, which corresponded to increased phosphorylation of ERK-1/2. Interestingly, IL-6 secretion and the production of both E-selectin and P-selectin were reduced in the presence of 10 μ M DS-SILY₂₀, even in the presence of the potent pro-inflammatory cytokine platelet-derived growth factor (PDGF). In hyperplastic and quiescent EC-SMC co-cultures, DS-SILY₂₀ treatment reduced the secretion of IFN- γ , IL-1 β , IL-6, and TNF- α , corresponding to a 23% decrease in p38 phosphorylation. E-selectin and P-selectin expression was further reduced following DS-SILY₂₀ treatment in both co-culture models. These results indicate that DS-SILY₂₀ promotes EC health and that this decorin mimic could serve as a potential therapeutic to promote vessel healing following percutaneous coronary intervention (PCI).

Keywords

Decorin; Endothelial Cells; Vascular Smooth Muscle Cells; Co-Culture; Proteoglycan; Dermatan Sulfate

1. Introduction

In healthy vasculature, the intact endothelium serves as a first line of defense, acting as a selective barrier for biochemical signals, regulating inflammation, and playing an important role in guiding smooth muscle cell (SMC) behavior to maintain proper vasodilation and the

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Conflict of Interest

Alyssa Panitch owns greater than 5% of Symic Biomedical, a company planning to enter into an agreement to license this technology from Purdue Research Foundation. This does not alter the authors' adherence to all the Journal of Tissue Engineering and Regenerative Medicine's policies on sharing data and materials.

suppression of intimal hyperplasia. Following percutaneous coronary intervention (PCI) procedures, the endothelial cell (EC) monolayer is stripped, exposing an underlying collagenous matrix and initiating coagulation and inflammatory cascades that trigger thrombus formation and stimulate SMC proliferation, migration, and extracellular matrix (ECM) deposition, ultimately resulting in restenosis (Amento et al. 1991, Farndale 2006, Hanke et al. 1990). While current therapies including both paclitaxel and the sirolimus analogs, widely used on drug-eluting stents, work to inhibit thrombi formation and halt maladaptive SMC activity, the non-specific effect of these SMC-targeted cytotoxins inhibits EC proliferation and migration, which is associated with incomplete vessel healing *in vivo* (Matter et al. 2006, Parry et al. 2005).

The regrowth of ECs and reestablishment of EC-SMC contact remain critical factors to ensure proper vessel healing (Scottburden and Vanhoutte 1993). Not only does re-endothelialization attenuate the need for systemic antiplatelet therapies, EC-SMC communication regulates SMC phenotype. Co-culture of ECs with SMCs inhibits the spontaneous transition of SMCs from a contractile to synthetic phenotype *in vitro* (Campbell and Campbell 1986, Ziegler et al. 1995). The effect of EC-SMC contact is not limited to regulating SMC behavior; ECs demonstrate a more elongated shape and exhibit enhanced coverage when cultured on quiescent SMCs as compared to on hyperplastic SMCs (Wallace et al. 2007).

The effect of cell-cell and cell-ECM contact, as well as cues from soluble factors, can differentially regulate EC and SMC behavior (Heydarkhan-Hagvall et al. 2003, Lilly 2014). ECs and SMCs remain targets for many of the pro-inflammatory factors released from activated inflammatory cells and platelets, which aid in regulating the phenotypic state of these cell types (Amento et al. 1991). One such factor, platelet-derived growth factor (PDGF), which has been associated with the onset of intimal hyperplasia in arterial-injury models (Jawien et al. 1992), activates intracellular transduction pathways in both ECs and SMCs. While PDGF stimulates proliferation and migration in SMCs, its function with respect to regulating EC behavior is more complex. PDGF has been found to encourage EC migration, yet also promote their quiescence (Dardik et al. 2005, Jiang et al. 1996, Marx et al. 1994, Matsumoto et al. 1999).

Further, glycosaminoglycans (GAG) and proteoglycans influence EC and SMC phenotypic states. GAG secretion from confluent EC monolayers prevented the phenotypic transition of quiescent SMCs *in vitro*, serving as a possible mechanism for modulating both SMC phenotype and proliferation. The exogenous addition of GAGs, such as heparin, and proteoglycans, decorin and perlecan, to proliferative SMCs also alters cell behavior, resulting in the transition to a more quiescent phenotype (Nili et al. 2003, Paka et al. 1999, Reilly et al. 1986). Moreover, our lab has developed a decorin mimic, DS-SILY₂₀, which modulates SMC migration, protein synthesis, cytokine secretion, and vascular injury marker production in both proliferative and quiescent SMCs *in vitro*, leading to reduced intimal hyperplasia in DS-SILY₂₀-treated vessels following PCI-related injury in Ossabaw swine (Scott et al. 2013).

While these macromolecules induce SMCs towards a more quiescent phenotype, the addition of GAGs and proteoglycans also enhances EC proliferation and migration *in vitro* (Fiedler et al. 2008, Rasente et al. 2012). For example, both low molecular mass dermatan sulfate and the proteoglycan decorin have been found to support EC proliferation and migration. Thus, the development of anti-restenotic therapeutics mimicking the natural regeneration prowess of GAGs could serve to both promote re-endothelialization of the injured vessel wall and inhibit SMC phenotype transition during this reparative process.

Here, we look to examine effects of DS-SILY₂₀ on ECs and on EC-SMC co-cultures. We have previously shown that media conditions and EC density, or monolayer formation, were critical to regulating SMC phenotype (Chaterji et al. 2010). Using the culture models described herein, we are able to further develop an understanding of how DS-SILY₂₀ will affect healthy and diseased blood vessels. We demonstrate the ability of DS-SILY₂₀ to enhance EC proliferation and migration, while attenuating pro-inflammatory cytokine secretion and the production of vascular injury markers, even in the presence of PDGF. Moreover, we utilize our *in vitro* models, including EC monolayers, hyperplastic EC-SMC co-cultures, and quiescent EC-SMC co-cultures, to emphasize the impact of cell-cell signaling in culture systems.

2. Materials and Methods

2.1 DS-SILY₂₀ Synthesis

The decorin mimic (DS-SILY₂₀) was synthesized as previously described (Paderi et al. 2011). Briefly, carboxyl groups present on the backbone of dermatan sulfate (DS, MW 46,275 Da, Celsus Laboratories) were oxidized via standard periodate oxidation to form aldehyde moieties. Oxidized DS was then covalently coupled to the heterobifunctional crosslinker *N*-[*b*-maleimidopropionic acid] hydrazide, trifluoroacetic acid salt (BMPH, Thermo Fisher Scientific) in phosphate buffered saline (PBS). The collagen-binding peptide sequence RRANAALKAGELYKSILYGC (noted as SILY, Genscript), derived from the platelet receptor to type I collagen, was conjugated to the DS-BMPH compound; specifically, the thiol group on the cysteine amino acid reacted with the maleimide group of BMPH to form a thioether bond. Purifications were performed at each step by size exclusion chromatography and the number of attached peptides was determined by the consumption of BMPH in the second reaction step. The final product DS-SILY_{*n*}, where *n* indicates the number of attached SILY peptides, was purified in ultrapure H₂O, lyophilized and stored at −20°C until use.

2.2 Cell Culture

2.2.1 Endothelial Cell Monocultures—Human aortic ECs (Invitrogen) were cultured in Media 200 (M200, Invitrogen), supplemented with (all from Invitrogen) 2% fetal bovine serum (FBS), 1 µg/mL hydrocortisone, 10 ng/mL epidermal growth factor (EGF), 3 ng/mL basic fibroblast growth factor (FGF), and 10 µg/mL heparin. Unless otherwise noted, cells were initially seeded at 3×10⁵ cells/cm² in Ibidi angiogenesis µ-slide (Ibidi) and allowed to proliferate for 24 hr to establish a confluent monolayer, prior to the application of treatments

to the EC cultures (Figure 1A). Cells were used between passage numbers 3 and 8 for all assays and maintained at 37°C with 5% CO₂.

2.2.2 Endothelial Cell-Smooth Muscle Cell Co-Cultures—To better mimic the physiology of the vessel wall and further investigate the effects of therapeutics on ECs in a more biologically relevant manner, two EC-SMC co-culture models, designed to mimic either healthy or diseased vasculature, were established (Figure 1B). ECs were cultured as stated previously. Human coronary artery SMCs (Invitrogen) were cultured in Media 231 (M231, Invitrogen), supplemented with (all from Invitrogen) 4.9% FBS, 2 ng/mL FGF, 0.5% EGF, 5 ng/mL heparin, 5 µg/mL insulin, and 0.2 µg/mL bovine serum albumin. SMCs were seeded at 5×10⁴ cells/cm² in Ibidi angiogenesis µ-slide and allowed to proliferate for 24 hr to allow the formation of multilayered cell constructs. Media was removed and cultures were treated either with proliferative media, as described above, or contractile media to induce a quiescent phenotype, for 24 hr. Previously, we demonstrated that the addition of contractile media, consisting of M231 supplemented with 1% FBS and 30 µg/mL heparin, induced SMCs to transition from a proliferative state to a more differentiated, contractile state due to low serum and introduction of heparin (Chaterji et al. 2010). After 24 hr, ECs were seeded at 3×10⁵ cells/cm² in co-culture media, consisting of M200 supplemented with 2% FBS, 1 µg/mL hydrocortisone, and 30 µg/mL heparin, to either the hyperplastic or quiescent SMC sublayer. ECs formed a monolayer atop of the SMC sublayers after 24 hr and treatments were then applied to the co-cultures. All cells were used between passage numbers 3 and 8 for all assays and maintained at 37°C with 5% CO₂.

2.3 Co-Culture Visualization

Cultures were fixed in 4% formaldehyde for 30 min, permeabilized with 0.5% TritonX-100 (Sigma) in PBS for 20 min, and blocked in 3% bovine serum albumin (BSA) in PBS for 1 hr. Detection of α-smooth muscle actin (αSMA), calponin, and von Willebrand Factor (vWF) in cultures was then performed in sequence. To identify SMCs, cultures were incubated with mouse anti-human αSMA (Invitrogen, 1:200 dilution) for 90 min with agitation, followed by incubation goat anti-mouse AlexaFluor® 488 (Invitrogen, 3 µg/mL). Calponin, a marker of SMC differentiation, was labeled using mouse anti-human calponin (Dako, 1:50 dilution) for 90 min with agitation, followed by incubation with goat anti-mouse AlexaFluor® 546 (Invitrogen, 3 µg/mL). Finally, vWF, an EC marker, was identified via mouse anti-human vWF (Dako, 1:25 dilution) for 90 min with agitation and goat anti-mouse AlexaFluor® 647 (Invitrogen, 3 µg/mL). Samples were washed for 20 min with agitation between each step to ensure removal of the previous antibody. Samples were visualized using a Nikon AR1 Multiphoton confocal microscope with 60x objective. Scans were completed with a xy area of 1028 µm² and one stack, 50 µm (1 µm per step) in the z-direction, was taken at ten separate locations in each culture.

2.4 Metabolic Activity

Cultures were incubated in the presence of 0, 0.01, 0.1, 1, or 10 µM DS-SILY₂₀, with and without 10 ng/mL PDGF, for 24 hr. The metabolic activity of the cells was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). Briefly, media was mixed with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-

sulfophenyl)-2H-tetrazolium, inner salt (MTS) and cultures were re-incubated for 2 hr at 37°C with 5% CO₂. The media containing MTS was then transferred into a 96-well plate and absorbance at 490 nm was measured.

2.5 Cell Viability

Cultures were incubated in the presence of 0, 0.01, 0.1, 1, or 10 μ M DS-SILY₂₀, with and without 10 ng/mL PDGF, for 24 hr. To test cytotoxicity of DS-SILY₂₀, EC viability was analyzed using LIVE/DEAD Viability/Cytotoxicity Assay Kit (Invitrogen) according to the manufacturer's instruction. Briefly, cultures were rinsed with PBS following treatment and 50 μ L of mixed solution of 2 μ M Calcein AM and 4 μ M ethidium homodimer-1 was added directly to cells. Following incubation for 30 min at room temperature, fluorescent intensity of the cultures was assessed via spectrophotometer to determine cell viability.

2.6 EC Proliferation

Cultures were incubated in the presence of DS-SILY₂₀ with or without 10 ng/mL PDGF for 24 hr. Cells were nuclei stained with Hoechst 33342 and absorbance was measured via spectrophotometer to determine the effect of DS-SILY₂₀ and PDGF on cell proliferation.

2.7 EC Migration

Fibrillar collagen was coated in wells of 96-well Oris Cell Migration Kit (Platypus Technologies, Madison). Stoppers were inserted into the plate to block an inner circular portion of the well. ECs were seeded at 5×10^3 cells/well and grown to confluence in the outer portion of the well. After 48 hr, the stoppers in the wells were removed and ECs were stained with cell tracker green (Invitrogen). To determine the effects of treatments on migration alone, ECs were dosed with 25 μ g/mL mitomycin C (Sigma) for 30 min to inhibit proliferation. Various DS-SILY₂₀ concentrations, solubilized in M200 + LSGS, were then incubated on the exposed collagen surface in the inner portion of the well for 15 min at 37°C. Unbound DS-SILY₂₀ was rinsed from the surface and cell media, with or without 10 ng/mL PDGF, was returned to the wells. ECs were allowed to migrate from the outer to the inner portion of the wells for 48 hr. At 48 hr, fluorescence measurements of the center of each well were measured using a mask provided with the migration kit such that only the treated inner circular portion of the well was measured.

2.8 Pro-Inflammatory Cytokine Expression

Cells were incubated in the presence of DS-SILY₂₀ with or without 10 ng/mL PDGF for 24 hr. Media was removed from the cultures and a Pro-Inflammatory I kit (Meso Scale Discovery) was used to analyze cytokine production from EC monolayers, hyperplastic co-cultures, and quiescent co-cultures according to manufacturer's instructions. Briefly, plates were warmed to room temperature and incubated with 25 μ L of samples and standards for 2 hr at room temperature with vigorous shaking. The detection antibody was then added to the plate and incubated for 2 hr at room temperature with vigorous shaking. After washing three times with PBS with 0.05% Tween-20, 2X read buffer was added to the plate and imaged using a Sector Imager 2400A (Meso Scale Discovery). The pro-inflammatory markers interferon- γ (IFN- γ), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis

factor- α (TNF- α) were examined in this study. Data were analyzed using the MSD Discovery Workbench Software and cytokine production from cultures was normalized to cell viability.

2.9 Production of Vascular Injury Markers

Following treatment with DS-SILY₂₀ with or without 10 ng/mL PDGF for 24 hr, cells were washed twice with ice cold PBS and solubilized in lysis buffer (9 M urea, 4% CHAPS, and phosphatase inhibitor cocktail-1 in Millipore water). Lysates were processed at 4°C for 30 min prior to centrifugation for 20 min at 18,000×g to remove membrane components. A BCA assay protein kit (Pierce) was used to quantify total protein. A Vascular Injury Marker I kit (Meso Scale Discovery) was used to analyze E-selectin, P-selectin, and thrombomodulin production from EC monolayers, hyperplastic co-cultures, and quiescent co-cultures according to manufacturer's instructions. Briefly, plates were warmed to room temperature and incubated with 10 μ L of samples and standards for 2 hr at room temperature with vigorous shaking. Following gentle rinsing of the wells, the detection antibody was then added and incubated for 1 hr at room temperature with vigorous shaking. After washing three times with PBS with 0.05% Tween-20, 2X Read buffer was added to the plate and imaged using a Sector Imager 2400A. Data were analyzed using the MSD Discovery Workbench Software.

2.10 MAPK Phosphorylation

The phosphorylation of mitogen-activated protein kinases (MAPK), including extracellular signal-related kinase (ERK) and p38 MAPK (p38), was examined. Cells were incubated in the presence of DS-SILY₂₀ with or without 10 ng/mL PDGF for 10 prior to washing with ice cold tris buffered saline (TBS) and solubilized in lysis buffer (150 mM NaCl, 20 mM Tris, 1 mM EDTA, 1 mM EGTA, 1% Triton-X-100, plus protease inhibitors and phosphatase inhibitors). Lysates were processed at 4°C for 30 min prior to centrifugation for 20 min at 18,000×g to remove membrane components. Phospho-p38 (Thr180/Tyr182) and phospho-ERK-1/2 (Thr/Tyr: 202/204; 185/187) levels were evaluated using the MAP Kinase Whole Cell Lysate kit (Meso Scale Discovery); Total p38 and total ERK-1/2 were determined via MAP Kinase (Total Protein) Whole Cell Lysate Kit (Meso Scale Discovery), according to manufacturer's instructions. Briefly, plates were warmed to room temperature and incubated with 25 μ L of samples for 3 hr at room temperature with vigorous shaking. Following gentle rinsing of the wells, the detection antibody was then added and incubated for 1 hr at room temperature with vigorous shaking. After washing three times with TBS, 2X read buffer was added to the plate and imaged using a Sector Imager 2400A. Data were analyzed using the MSD Discovery Workbench Software. The relative amount of phosphorylated p38 and ERK-1/2 were normalized to total p38 and ERK-1/2 for each sample.

2.11 PDGFR β Phosphorylation

Cells were incubated in the presence of DS-SILY₂₀ with and without 10 ng/mL PDGF for 60 min prior to washing with ice cold TBS and the addition of lysis buffer prior to washing with ice cold TBS and solubilized in lysis buffer (1% NP-40 Alternative, 20 mM Tris, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM activated sodium orthovanadate, 10 μ g/mL Aprotinin, and 10 μ g/mL Leupeptin). Lysates were processed at 4°C for 30 min prior to

centrifugation for 5 min at 2000×g to remove membrane components. Sandwich ELISAs (all from R&D systems) were utilized to measure total PDGF receptor β (PDGFR β) and total phosphotyrosine PDGFR β . All assays were performed following the manufacturer's protocol.

2.12 Statistical Analysis

Results are expressed as means $\pm$ standard error. Statistical analysis was performed using SAS software (SAS Institute). All results were analyzed using ANOVA with Tukey HSD post-hoc test. The threshold for statistical significance was set at $p < 0.05$.

3. Results

3.1 Co-Culture Visualization

Co-cultures were examined for α SMA, calponin, and vWF (Figure 1C-F). Prior to the addition of ECs, proliferative SMCs (Figure 1C) were positive for α SMA, while staining minimally for calponin, a marker of SMC differentiation. Quiescent SMCs (Figure 1E) stained intensely for both α SMA and calponin. ECs were then detected in both the hyperplastic (Figure 1D) and quiescent (Figure 1F) co-cultures by probing for vWF.

3.2 EC and EC-SMC Co-Culture Metabolic Activity

EC metabolic activity was assessed via the CellTiter 96 AQueous One Solution Cell Proliferation Assay. Low concentrations of DS-SILY₂₀ did not alter the metabolic activity of ECs, compared to no treatment controls (Figure S1A, Supporting Information). However, metabolic activity in ECs significantly decreased following treatment with 10 μ M DS-SILY₂₀ ($p < 0.02$). The addition of 10 ng/mL PDGF alone did not influence the metabolic activity of ECs ($p = 0.15$). Similar to results seen when ECs were treated with DS-SILY₂₀ alone, a significant decrease in metabolism was observed when PDGF-stimulated ECs were treated with 10 μ M DS-SILY₂₀ ($p < 0.002$). However, no changes in metabolic activity were observed in PDGF-treated cultures stimulated with lower concentrations of DS-SILY₂₀.

DS-SILY₂₀, at every concentration tested, did not alter the metabolic activity of either the hyperplastic or quiescent co-cultures, compared to no treatment controls (Figure S1B & C, Supporting Information). Further, the metabolic activity of the co-cultures was not impacted via PDGF stimulation alone or with the subsequent addition of DS-SILY₂₀.

3.3 Viability of ECs and EC-SMC Co-Cultures

To further explore the impact of DS-SILY₂₀ on EC monolayers, hyperplastic co-cultures, and quiescent co-cultures, a cell viability assay was completed (Figure S1D-F, Supporting Information). After 24 hr of stimulation, 88.9% $\pm$ 4.4% of ECs were found viable in no treatment control cultures. Both hyperplastic and quiescent co-cultures exhibited similar viability compared to ECs, where 87.0% $\pm$ 1.2% and 87.2% $\pm$ 1.6% of cells remained viable after 24 hr of treatment, respectively. The addition of any concentration of DS-SILY₂₀, with or without added PDGF, did not significantly alter cell viability in EC monolayer cultures or in either co-culture system.

3.4 PDGFR β Phosphorylation in ECs

To assess the ability of DS-SILY₂₀ to influence PDGF signaling in EC monolayers, phosphorylation of PDGFR β was investigated. The addition of 10 μ M DS-SILY₂₀ to EC monolayers significantly decreased phospho-PDGFR β compared to no treatment controls (Figure 2A; $p < 0.02$). Conversely, phospho-PDGFR β levels were significantly increased in EC monolayers with PDGF treatment ($p < 0.05$). However, the addition of DS-SILY₂₀ to PDGF-stimulated ECs resulted in significantly decreased phosphorylation of PDGFR β , compared to both EC cultures stimulated with PDGF alone ($p < 0.05$) and to no treatment control cultures ($p < 0.02$).

3.5 EC Proliferation is Enhanced By DS-SILY₂₀

EC proliferation was assessed by examining changes in absorbance of nuclei-stained cells following treatment for 24 hr. The addition of DS-SILY₂₀ increased EC proliferation in a dose-dependent manner, where the addition of 10 μ M DS-SILY₂₀ resulted in a 1.5-fold increase in proliferation compared to no treatment controls (Figure 2B; $p < 0.05$). PDGF also significantly increased proliferation in monolayer EC cultures ($p < 0.05$). The co-addition of PDGF and low concentrations of DS-SILY₂₀ did not result in a synergistic increase in proliferation; rather, proliferation in PDGF-stimulated ECs treated with 0.01 or 0.1 μ M DS-SILY₂₀ remained similar to that of cultures treated with PDGF alone. However, significantly increased proliferation was observed in PDGF-stimulated ECs treated with 1 μ M DS-SILY₂₀, as compared to ECs treated with PDGF alone ($p < 0.05$). Interestingly, EC proliferation was observed to decrease significantly when cells were simultaneously treated with 10 μ M DS-SILY₂₀ and 10 ng/mL PDGF ($p < 0.02$).

3.6 DS-SILY₂₀ encourages EC Migration

The effect of DS-SILY₂₀ and PDGF on EC migration across collagen-coated surfaces was examined via Oris Cell Migration Kit. Mitomycin C was utilized to growth-arrest ECs prior to treatments, thus isolating cell migration measurement with each treatment condition. A dose dependent increase in EC migration was observed with corresponding increases in DS-SILY₂₀ concentrations, ultimately culminating in a ~2.2-fold increase in migration for cultures treated with 10 μ M DS-SILY₂₀ (Figure 2C; $p < 0.05$). PDGF stimulation also significantly enhanced EC migration by 40.3% ($p < 0.05$). Similar to the trend observed in ECs treated with DS-SILY₂₀ alone, the addition of DS-SILY₂₀ to PDGF-stimulated cultures resulted in a dose-dependent increase in migration. Migration was increased ~3-fold in PDGF-stimulated ECs treated with 10 μ M DS-SILY₂₀ ($p < 0.05$).

3.7 DS-SILY₂₀ Attenuates IL-6 Expression in ECs

Following exposure to DS-SILY₂₀ and PDGF treatments for 24 hr, expression of IFN- γ , IL-1 β , IL-6, and TNF- α from EC monolayers was examined via MSD Sector Imager. Examination of control, non-treated EC monolayers revealed that ECs exhibited 117.2 ± 10.4 pg/mL of IL-6 (Figure 2D). The addition of 10 ng/mL PDGF to ECs did not alter IL-6 production compared to no treatment controls ($p = 0.35$). While no reduction in IL-6 production was observed when ECs were treated with low concentrations of DS-SILY₂₀, the addition of 10 μ M DS-SILY₂₀ resulted in significantly decreased IL-6 expression ($p < 0.01$),

where reductions of ~50%, were observed in both PDGF-stimulated and unstimulated EC cultures. IFN- γ , IL-1 β , and TNF- α produced by EC monolayers remained undetectable, likely produced at minute concentrations.

3.8 Effect of DS-SILY₂₀ on EC Vascular Injury Marker Expression

To determine the effect of DS-SILY₂₀ and PDGF on vascular injury markers, E-selectin, P-selectin, and thrombomodulin produced by EC monolayers *in vitro* were analyzed via an MSD Sector Imager (Figure 3). Control non-treated ECs monolayers exhibited 0.2 pg/mg, 0.6 pg/mg, and 23.6 pg/mg of E-selectin, P-selectin, and thrombomodulin, respectively. Production of all three vascular injury markers decreased in EC monolayers with increasing DS-SILY₂₀ concentrations, ultimately culminating in a 3.2, 1.4, and 1.4-fold reduction in E-selectin ($p<0.05$), P-selectin ($p<0.05$), and thrombomodulin ($p<0.01$) expression, respectively. The addition of 10 ng/mL PDGF to ECs did not alter E-selectin production compared to no treatment controls ($p=0.65$). However, PDGF induced both P-selectin ($p<0.05$) and thrombomodulin ($p<0.05$) expression in EC monolayers. The addition of DS-SILY₂₀ to PDGF-stimulated EC monolayers resulted in significantly decreased E-selectin ($p<0.001$), P-selectin ($p<0.01$), and thrombomodulin ($p<0.001$) production, where expression was decreased by ~60%, 56%, and 38%, respectively, following treatment with 10 μ M DS-SILY₂₀.

3.9 Phosphorylation of ERK and p38 MAPK in ECs

MAPKs, including ERK and p38, are important intracellular transduction pathways involved in vascular remodeling and disease (Force et al. 1996, Xu et al. 1996). Phosphorylation of ERK-1/2 has previously been correlated with increased EC proliferation and migration, and several studies have correlated p38 phosphorylation with increased inflammatory cytokine expression (Cai et al. 2006, Goetze et al. 2002, Hashimoto et al. 1999). Thus, we sought to correlate the changes observed in proliferation and migration (Figure 2B & C) or cytokine expression (Figures 2D) with ERK-1/2 or p38 phosphorylation levels. To determine the relative amount of phosphorylated ERK-1/2 (pERK-1/2) and p38 (pp38), EC monolayers were stimulated with DS-SILY₂₀, with or without 10 ng/mL PDGF, for 10 min. Cell lysates were then analyzed via MSD Sector Imager to determine the relative phosphorylation levels of the intracellular signaling molecules (Figure 4).

After 10 min of stimulation, a dose-dependent increase in relative pERK-1/2 levels occurred with increasing concentrations of DS-SILY₂₀ (Figure 4A). ERK-1/2 phosphorylation was increased in EC monolayers stimulated with 10 ng/mL PDGF compared to no treatment controls ($p<0.02$). Further, the addition of increased DS-SILY₂₀ concentrations corresponded to a dose-dependent increase in relative pERK-1/2 levels in PDGF-stimulated EC monolayers.

The addition of DS-SILY₂₀ resulted in a dose-dependent inhibition of relative pp38 levels in monolayer ECs compared to no treatment controls, after 10 min of stimulation (Figure 4B). PDGF did not alter the phosphorylation of p38 in EC monolayers; however, the addition of DS-SILY₂₀ to PDGF-stimulated ECs resulted in a similar dose-dependent decrease in pp38.

3.10 Cytokine Production in EC-SMC Co-Cultures

Following DS-SILY₂₀ and PDGF treatments for 24 hr, expression of IFN- γ , IL-1 β , IL-6, and TNF- α from EC-SMC co-cultures was examined via MSD Sector Imager, in both the hyperplastic and quiescent co-cultures (Figure 5). Examination of control, non-treated cultures revealed that hyperplastic co-cultures exhibited increased levels of IL-1 β compared to quiescent co-cultures ($p<0.001$); however, the two cultures produced similar levels of IFN- γ ($p=0.23$) IL-6 ($p=0.87$), and TNF- α ($p=0.91$) (Figure 5C & D).

The effect of DS-SILY₂₀ on the production of the four pro-inflammatory cytokines was evaluated in both hyperplastic and quiescent co-cultures. For hyperplastic co-cultures, a dose-dependent decrease of pro-inflammatory cytokine production was observed as the concentration of DS-SILY₂₀ increased. The addition of 10 μ M DS-SILY₂₀ decreased IL-1 β ($p<0.001$) and IL-6 ($p<0.01$) secretion in hyperplastic co-cultures by ~55% and 66%, respectively. Further, both IFN- γ ($p<0.001$) and TNF- α ($p<0.02$) secretions were reduced by approximately 98% following treatment with 10 μ M DS-SILY₂₀ in hyperplastic co-cultures. A significant reduction in IFN- γ expression was exhibited in quiescent co-cultures with 1 and 10 μ M DS-SILY₂₀ treatment, while TNF- α expression was significantly decreased with the addition of 10 μ M DS-SILY₂₀. Interestingly, significant decreases in IL-1 β and IL-6 production were achieved at all concentrations of DS-SILY₂₀ tested.

The addition of 10 ng/mL PDGF significantly increased production of IFN- γ ($p<0.02$), IL-6 ($p<0.01$), and TNF- α ($p<0.05$) in hyperplastic co-cultures, as well as IFN- γ ($p<0.05$) and TNF- α ($p<0.02$) in quiescent co-cultures, compared to no treatment controls. However, a general trend was observed such that as the concentration of DS-SILY₂₀ increased, cytokine production in PDGF-stimulated hyperplastic co-cultures decreased. Significant reductions in IFN- γ ($p<0.002$), IL-1 β ($p<0.01$), IL-6 ($p<0.001$), and TNF- α ($p<0.001$) expression were observed at the highest levels of DS-SILY₂₀ tested in hyperplastic co-cultures, compared to co-cultures treated with PDGF alone. Interestingly, quiescent co-cultures exhibited increased IFN- γ and IL-6 production following treatment with 10 ng/mL PDGF and either 0.01 or 0.1 μ M DS-SILY₂₀. However, at high concentrations of DS-SILY₂₀, expression of IFN- γ ($p<0.02$) and IL-6 ($p<0.001$) in PDGF-stimulated quiescent co-cultures was significantly reduced compared to co-cultures stimulated with PDGF alone. Moreover, while TNF- α and IL-1 β production in PDGF-stimulated quiescent co-cultures was not altered when cultures were treated with low concentrations of DS-SILY₂₀, significant reductions in expression of TNF- α ($p<0.05$) and IL-1 β ($p<0.05$) were only observed at 10 μ M DS-SILY₂₀.

3.11 Vascular Injury Marker Expression in Co-Cultures

To determine the effect of DS-SILY₂₀ and PDGF on vascular injury markers, E-selectin, P-selectin, and thrombomodulin produced by EC-SMC co-cultures *in vitro* were analyzed via MSD Sector Imager (Figure 6). Control hyperplastic co-cultures exhibited 0.2 pg/mg E-selectin; however, quiescent co-cultures produced significantly less E-selectin when compared to hyperplastic co-cultures (Figure 6A & B; $p<0.05$). Hyperplastic and quiescent co-cultures produced similar levels of P-selectin (Figure 6C & D; $p=0.51$) and thrombomodulin (Figure 6E & F; $p=0.42$). While thrombomodulin production was not altered in co-cultures following DS-SILY₂₀ treatment, E-selectin ($p<0.05$) and P-selectin

($p < 0.05$) expression was significantly decreased in both co-cultures following treatment with 10 μM DS-SILY₂₀. Interestingly, treatment with low concentrations of DS-SILY₂₀ increased production of P-selectin in the hyperplastic co-culture model ($p < 0.05$); however, this increase was not mirrored in the quiescent co-culture model.

PDGF was added, both with and without DS-SILY₂₀ treatment, to assess its impact on vascular injury marker production in co-culture models. P-selectin ($p < 0.05$) and thrombomodulin ($p < 0.05$) were significantly increased in both the hyperplastic and quiescent co-cultures following PDGF stimulation. PDGF did not stimulate E-selectin expression in either co-culture system. However, the addition of 10 μM DS-SILY₂₀ to PDGF-stimulated hyperplastic or quiescent co-cultures resulted in decreased expression of E-selectin ($p < 0.05$), P-selectin ($p < 0.05$), and thrombomodulin ($p < 0.05$).

4. Discussion

Cytotoxins currently utilized to minimize maladaptive cell behavior following PCI are non-selective, and the goal for functional vascular healing should be a development of new active ingredients that promote EC growth while inhibiting unregulated SMC behavior. Previously, we have demonstrated that DS-SILY₂₀ modulates SMC migration, protein synthesis, cytokine secretion, and vascular injury marker production in both proliferative and quiescent SMCs (Scott et al. 2013, Scott and Panitch 2014). Conversely, in this work, we reveal that DS-SILY₂₀ supports EC proliferation and migration *in vitro* (Figure 2B & C). These findings are in agreement with previous reports detailing that both low molecular weight dermatan sulfate and native decorin support EC proliferation and migration (Fiedler et al. 2008, Rasente et al. 2012). Consistent with the increased proliferation and migration observed with PDGF stimulation in these studies, the relative levels of pERK-1/2, known to be associated with proliferation and migration, also increased (Figure 4A) (Qi et al. 2011).

While PDGF had little to no effect on the stimulation of proliferation in ECs treated with DS-SILY₂₀ concentrations up to 1 μM , the combination of 10 μM DS-SILY₂₀ with 10 ng/mL PDGF significantly decreased proliferation as compared to untreated ECs or ECs treated with either PDGF or 10 μM DS-SILY₂₀ alone. This proliferation decrease corresponded to decreases in both cell metabolism (Figure S1A, Supporting Information) and the relative ratios of phosphorylated PDGF receptors in ECs stimulated with both 10 μM DS-SILY₂₀ and 10 ng/mL PDGF (Figure 2A). It is likely that the decrease in metabolism is directly related to the decrease in cell proliferation; however, as no measureable changes in cell viability were observed in EC monolayers following treatment, the decrease in EC metabolism with 10 μM DS-SILY₂₀ did not provide any cause for concern. Further, the induction of a quiescent EC phenotype has been linked to the downregulation of PDGF receptors, but the underlying mechanism for this observation remains unknown (Marx et al. 1994). Interestingly, exposure to high concentrations of DS-SILY₂₀ and 10 ng/mL PDGF synergistically enhanced EC migration, a mechanism independent of proliferation and metabolism and likely required for repopulating denuded vessels.

Following vessel injury, damaged ECs participate in the inflammatory cycle by producing and secreting a range of pro-inflammatory factors, in response to mechanical and chemical

stimuli (Cines et al. 1998, Nugent et al. 2000). Though shown to be key players during restenosis, IFN- γ , IL-1 β , and TNF- α production in EC monolayers remained undetected, likely expressed at concentrations below the detection threshold. However, IL-6, which plays roles in both inflammation and fibrosis, was detected (Figure 2D). Consistent with studies demonstrating that decorin attenuates cytokine production in dysfunctional endothelium (Nelimakka et al. 2001), we reveal here that DS-SILY₂₀ reduces IL-6 secretion from ECs, both with and without PDGF stimulation. As we have previously demonstrated, PDGF exhibits a high affinity for DS-SILY₂₀ (Scott and Panitch 2014). Taken together, these results provide evidence that DS-SILY₂₀ is able to modulate vascular EC function even in the presence of the pro-inflammatory mediator PDGF, which interact strongly with the DS-SILY₂₀.

We further investigated the effect of DS-SILY₂₀ on pro-inflammatory cytokine production in our two co-culture models in order to gain a better understanding of the potential effect of DS-SILY₂₀ on vascular cell types in settings mimicking uninjured and post-PCI *in vivo* conditions. Similar to results obtained in previous studies, cytokine production was enhanced in co-cultures compared to EC monolayers due to the additive effect of SMC secretions (Chiu et al. 2007). Conversely, overall cytokine secretion in both of our EC-SMC co-cultures was decreased compared to our previous findings examining pro-inflammatory cytokine production from proliferative and quiescent SMC monocultures (Scott et al. 2013, Scott and Panitch 2014). We demonstrate here that quiescent EC-SMC co-cultures elicited decreased IL-1 β production compared to their hyperplastic counterparts (Figure 5C & D), emphasizing that quiescent SMCs can induce endothelium to present a less inflammatory surface. Taken together, these observations further demonstrate the importance of cell-cell contact, as well as cell phenotype, on the responsiveness of cells within EC-SMC culture models. Moreover, these findings highlight the importance of endothelial therapeutic targets, as complete, healthy endothelium suppresses platelet activation, SMC proliferation, and ECM secretion (Campbell and Campbell 1986, Freedman 2005).

DS-SILY₂₀ radically reduced cytokine production in a dose-dependent manner; however, more pronounced effects were observed in hyperplastic co-cultures compared to quiescent co-cultures, with high DS-SILY₂₀ concentrations mitigating cytokine expression to levels at or below that of quiescent co-cultures. Even in the presence of PDGF, DS-SILY₂₀ suppressed inflammatory cytokine expression in all culture formats, with the 10 μ M DS-SILY₂₀ concentration showing the greatest effect. This speaks to the utility of DS-SILY₂₀ as a potential therapeutic for PCI. Cultivation of a less hostile environment, without the recruitment of monocytes and other inflammatory cells, may provide for an enhanced healing environment. In addition to minimizing inflammatory cell recruitment, obtaining quiescent SMC conditions is important for maintaining an EC monolayer on SMCs (Chaterji et al. 2010). We have previously reported the ability of DS-SILY₂₀ to transition proliferative SMCs to a more quiescent phenotype (Scott et al. 2013), finding this transition to be further evident in our hyperplastic co-culture model following DS-SILY₂₀ treatment.

We further examined the effect of DS-SILY₂₀ on the expression of the vascular injury markers E-selectin, P-selectin, and thrombomodulin in our healthy and diseased co-culture models. Vascular injury marker analysis was performed using the lysate from the entire

population of cells, including both ECs and SMCs, in the EC-SMC co-cultures. However, as E-selectin and P-selectin secretion from SMC monocultures was not detected in our previous work, but is expressed by the EC monolayers (Figure 3), we hypothesize that the ECs primarily were responsible for expression of these two molecules in the co-culture systems. Cell adhesion molecules, such as E-selectin and P-selectin, are upregulated in dysfunctional endothelium and participate in the leukocyte-EC/platelet-EC interactions that lead to complications following PCI (Ahluwalia et al. 2004). It has previously been demonstrated that quiescent SMCs, and their apical ECM, induce the endothelium to present a less thrombotic surface (Wallace et al. 2007, Wallace and Truskey 2010). Our work further emphasizes this, demonstrating decreased E-selectin and P-selectin expression in our quiescent EC-SMC co-culture model, compared to EC monolayers alone. Thus, this model of a healthy vessel wall illustrates possible functions of the underlying quiescent SMCs on the thrombotic and inflammatory response of the endothelium. Further, P-selectin production was also reduced in hyperplastic EC-SMC co-cultures, as compared to EC monolayers, implicating the importance of cell-cell contact, independent of phenotype, towards directing the response of cells to their microenvironments.

The impact of EC-SMC contact on the extent to which ECs present a thrombotic surface is also evident in our thrombomodulin data. Expression of thrombomodulin, an EC membrane protein functioning as a key thrombin regulator in the blood coagulation cascade, was decreased in both co-culture models compared to EC monolayers alone, further demonstrating the ability of EC-SMC contacts to influence ECs to present a less thrombotic surface. Although an upregulation of thrombomodulin helps inhibit the coagulation cascade, a decrease in thrombomodulin expression by ECs (induced at high DS-SILY₂₀ levels), coupled with the decrease seen in P- and E-selectin may be an indication of endothelium health since in the absence of trauma and upregulated P-selectin, the endothelium naturally suppresses coagulation. The addition of high concentrations of DS-SILY₂₀ also decreased E-selectin, P-selectin, and thrombomodulin, even in the presence of PDGF, in all three culture models, linking the effects of this biomolecule to a healthy, intact endothelium.

In conclusion, we demonstrate in this work that DS-SILY₂₀ is able to induce proliferation and migration in ECs, while minimizing pro-inflammatory cytokine and vascular injury marker production. Our *in vitro* models, including EC monolayers, as well as both hyperplastic and quiescent EC-SMC co-cultures, further emphasize the importance of cell-cell signaling in culture systems. These results, coupled with our previous results demonstrating the ability of DS-SILY₂₀ to attenuate maladaptive SMC behavior, indicate that DS-SILY₂₀ could serve as potential drug to promote vessel healing following PCI and further investigation is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experimental design and visualization of cultures.

(A) ECs, initially seeded at 3×10^5 cells/cm², were treated with DS-SILY₂₀ for 24 hr before analysis. (B) Proliferative SMCs, initially seeded at 5×10^4 cells/cm², were either kept in a (i) proliferative phenotype or (ii) differentiated into a quiescent phenotype due to differences in media. After 24 hr, ECs were seeded upon the hyperplastic SMCs to obtain a hyperplastic co-culture or onto quiescent SMCs creating a quiescent co-culture, prior to treating the co-cultures with DS-SILY₂₀. (C-F) Representative images of (C) proliferative and (E) quiescent SMCs, as well as (D) hyperplastic and (F) quiescent co-cultures, stained for αSMA (green), calponin (red), and vWF (blue). Scale bars = 20 μm.

Figure 2. DS-SILY₂₀ regulates phosphorylation of PDGFRβ, proliferation, migration, and IL-6 production in ECs.

(A) Relative phosphorylated PDGFRβ produced in ECs treated with 10 μM DS-SILY₂₀ and 10 ng/mL PDGF. The relative amount of phosphorylated PDGFRβ was normalized to total PDGFRβ for each sample. (B) Proliferation of ECs in response to DS-SILY₂₀ treatment, with and without PDGF stimulation. (C) EC migration in response to DS-SILY₂₀ treatment, with and without PDGF stimulation. (D) EC production of IL-6 in response to DS-SILY₂₀ treatment, with and without PDGF stimulation. IL-6 production from cultures was normalized to cell viability. * represents significance from control non-treated cells (p<0.05); # represents significance PDGF-treated cultures. AFU: arbitrary fluorescence units. (N=3–6)

Figure 3. E-selectin, P-selectin, and thrombomodulin expression in ECs is regulated by DS-SILY₂₀. Expression of (A) E-selectin (B) P-selectin, and (C) thrombomodulin in ECs treated with DS-SILY₂₀, with and without PDGF stimulation. E-selectin, P-selectin, and thrombomodulin produced by cultured ECs was measured 24 hr post-treatment following cell lysis. * represents significance from control non-treated cells; # represents significance PDGF-treated cultures. (N=6–9)

Figure 4. DS-SILY₂₀ regulates ERK-1/2 and p38 phosphorylation in ECs.

Relative phosphorylated (A) ERK-1/2 and (B) p38 produced in ECs treated with DS-SILY₂₀, with and without 10 ng/mL PDGF. The relative amount of phosphorylated ERK and p38 was normalized to total ERK and total p38 for each sample, respectively. * represents significance from control non-treated cells; # represents significance from PDGF-treated cultures. (N=3)

Figure 5. DS-SILY₂₀ attenuates PDGF stimulated cytokine secretion in ECs.

Cytokine produced (A, C, E, G) hyperplastic and (B, D, F, H) quiescent co-cultures in response to DS-SILY₂₀ treatment, with and without PDGF stimulation. The amount of (A, B) IFN-γ, (C, D) IL-1β, (E, F) IL-6, and (G, H) TNF-α produced by co-cultures was measured 24 hr post-treatment. Cytokine production from cultures was normalized to cell viability. * represents significance from control non-treated cells, # represents significance from PDGF-treated cultures. (N=6)

Figure 6. DS-SILY₂₀ modulates E-selectin, P-selectin, and thrombomodulin expression in EC-SMC co-cultures.

Expression of (A, B) E-selectin (C, D) P-selectin, and (E, F) thrombomodulin in (A, C, E) hyperplastic and (B, D, F) quiescent co-cultures treated with DS-SILY₂₀, with and without PDGF stimulation. E-selectin, P-selectin, and thrombomodulin produced by co-cultures was measured 24 hr post-treatment following cell lysis. * represents significance from control non-treated cells; # represents significance PDGF-treated cultures. (N=6)