POTENTIAL OF AUTOLOGOUS CIRCULATING CD34+45- SOURCE CELLS TO INHIBIT SMOOTH MUSCLE CELL DEDIFFERENTIATION FOR INTRACRANIAL AND VASCULAR STENT APPLICATIONS

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Abstract-Balloon angioplasty ± stenting is often complicated by restenosis, initiated in part by endothelial denudation, leading to smooth muscle cell dedifferentiation and proliferation. These complications could possibly be mitigated by re-endothelialization of the stented vessel lumen including the stent surface using autologous cells. Circulating CD34+CD45- cells are the source cells of late-outgrowth endothelial progenitor cells and can be isolated by FDA-approved immuno-magnetic selection. Our pilot studies tested the hypotheses that the CD34+45- source cells could adhere to (Ti), the blood-contacting surface material of nitinolintracranial and vascular stents, and that the CD34+45- source cells on Ti would differentiate into endothelial cells with the potential to inhibit smooth muscle cell dedifferentiation. Our results show that the an average of 39.7± 6.0%, 19.7± 2.1%, 14.7± 6.8% ofsource cells remained adherent on Ti under laminal fluid flow shear stress of 10, 15, and 20dynes/cm^2 (n=3 for each shear stress). The cells differentiated into endothelial cells on Ti, shown by EC-characteristic cobblestone morphology, Di-Ac-LDL uptake, PECAM-1 positive staining and thrombomodulin expression. Lining bare Ti surfaces with a confluent monolayer of the CD34+45- cellssignificantly reduced the ability of platelets to adhere to those surfaces, with only 149.4 ± 178.0 platelets/mm^2 adhered to the cell-lined Ti surfaces, compared to 2372.9 ± 29.2 for the bare surfaces. Further, the CD34+45- source cells showed potential to inhibit SMC dedifferentiation as assessed by increased smooth muscle cell-specific differentiation marker □-SMA. Future *in vivo* studies are warranted to investigate the effect of CD34+45- source cells to regenerate damaged endothelium after angioplasty ± stenting by lining the blood-contacting surface of stents and potentially replacing endothelial cells that were sheared off from the vessel lumen.

Keywords: Endothelial Progenitor Cell, Stent, Smooth-muscle cell, Immuno-magnetic selection, α-SMA, Thrombomodulin

I. INTRODUCTION

Smooth muscle cell (SMC) proliferation plays a central role in the pathophysiology of hypertension and atherosclerosis. One of the principal approaches to treat the culprits of atherosclerosis is balloon angioplasty ± stenting. Angioplasty is complicated by restenosis, initiated in part by endothelial denudation. A lack of endothelium leads to further SMC proliferation and dedifferentiation. To reduce SMC dedifferentiation and proliferation, drug-eluting stents were developed. Yet, drug-eluting stents delay the regeneration of endothelium and increase the risk of late-stent thrombosis. To avoid these complications, it would be highly desirable to rapidly re-endothelialize the stented vessel lumen. Furthermore, rapidly endothelialized stent surfaces would reduce the risk of stent thrombosis and potentially reduce the need for antithrombotic medications, which are especially dangerous after intracranial stenting because they can cause cerebral hemorrhage. However, unless harvested surgically from a vessel, autologous endothelial cells (ECs) are not available An alternative approach to obtain ECs are by using capture stents coated with an antibody against CD34, which aims to capture endothelial progenitor cells *in vivo*. Yet, as an exclusive marker for endothelial progenitor cells has not yet been identified, anti-CD34 antibodies also recognize prothrombotic cells of the monocytic lineage, CD45+ cells.

It has been established that circulating CD34+CD45- cells are the source of endothelial progenitor cells, aka as late-outgrowth endothelial progenitor cells, which have all the phenotypic characteristics of true endothelial cells.[1] These blood-derived endothelial cells would be an easily accessible source of autologous ECs for therapeutic purposes, but only appear after 2-3 weeks of static *ex vivo* culture of the CD34+CD45- source population.[2] Fluid shear stress has recently been shown to induce the differentiation of a different cell population, circulating CD133+ angiogenic cells, towards an EC-like phenotype.[3] Similarly, EC-specific markers increased when embryonic stem cells cultured on collagen were subjected to fluid shear stress.[4,5]

Vascular (nitinol) stentsare commonly made of titanium (Ti) as their outermost blood-contacting surface and prior studies have shown that ECs adhere well to uncoated Ti surfaces due to the naturally formed titanium dioxide film.[6] In the present study, we tested the hypotheses that CD34+45- source cells

were able to adhere to titanium surface under physiological fluid shear stress, differentiate toward an endothelial cell phenotype and exhibit EC character such as inhibiting SMC dedifferentiation.

II. METHODS

Isolation of CD34+45- Source Cells

We utilized human umbilical cord blood (HUCB), obtained from the Duke University Cord Blood Bank according to our approved Institutional Review Board protocol. Blood was collected with Cord Blood Collection Units (Pall Corporation, NY), which contained 30-45% of citrate phosphate dextrose (CPD). For each ml of the blood and CPD solution, 20 USP of heparin (APP Pharmaceuticals, IL) was added. Mononuclear cells (MNCs) were collected via density gradient separation using a histopaque layer and, in the final step,resuspended in Miltenyi buffer to proceeding Miltenyi Immuno-Magnetic Selection (Dynabeads, Life Technologies).

Flow Cytometry to Test for Purity

CD45-34+ cells were labeled with anti-CD34 and anti-CD45 antibodies. Respective isotype controls were used. The fluorescent intensity was measured with a FACSCalibur flow cytometer (Becton–Dickinson, NJ). For each set of samples, the fluorescent intensity of the isotype control was compared with the fluorescent intensity of the test sample using CellQuest software (Becton Dickinson).

Adherence of CD34+45- Source Cells on Ti Under Shear Stress

The isolated CD45-34+ source cells were seeded on uncoated Ti surfaces for 15 minutes at 37°C, 5% CO_2. Following, the adherence of CD34+45- cells to Ti was investigated by using a parallel-plate flow chamber and flow circuit[7] (5 minutes, 37°C, 5% CO_2) at various physiological shear stresses: 10 dynes/cm^2 (58 mL/min), 15 dynes/cm^2 (87 mL/min) and 20 dynes/cm^2 (117 mL/min) in full growth EC medium, EGM-2 supplemented EBM-2 (Lonza, Basel). Quantification of adherent cells was done using the Cell Counter function of ImageJ software.

Differentiation of CD34+45- Source Cells to ECs on Ti

The isolated CD45-34+ cells were seeded on Ti surfaces and then cultured for 14 days (37°C, 5% CO_2) to investigate their differentiation toward an EC phenotype as characterized by their morphology, phenotypic and functional characteristics. The morphology of differentiated source cells was observed under phase contrast microscope at the end of 14 day culture. The cell phenotypic and functional properties on day 14 were characterized by performing a DiI-Ac-LDL assay (Biomedical Technologies, MA) and immunocytochemistry staining for PECAM-1 and thrombomodulin (Santa Cruz, CA).

Platelet Adhesion to Ti Lined with CD34+45- Source Cells

Human platelets were washed and suspended in a buffered saline glucose citrate solution (8.6 mM Na_2HPO_4, 1.6 mM KH_2PO_4, 0.12 M NaCl, 0.9 mM EDTA, 13.6 mM Na_3citrate, 11.1 mMglucose, at pH 7.1) A confluent monolayer of CD45-34+ cells was labeled with Cell Tracker Green, while a solution of 10^6 platelets was labeled with Cell Tracker Orange and then suspended (and activated) in 10 mL of 1:1 DMEM/Tyrodes buffer (20mM Hepes,137mM NaCl, 2.7 mMKCl, 1 mM $CaCl_2$, 11.9 mM $NaHCO_3$, 5.5 mM Glucose). 5 mL of this platelet suspension was added to either bare Ti or Source cell-coated Ti (CD45-34+ source cells were cultured for 14 days under static conditions on Ti), followed by 10 min incubation(37°C, 5% CO_2). Surfaces were rinsed 3 times with PBS and then imaged.

Co-culture of CD34+45- Source Cells and SMCs

SMCs were grown to confluence in SMC basal medium (SmBMsupplemented with SmGM-2 singleQuots, Clonetics) and then induced to a quiescent state via 2 days in SMC quiescent medium composed of DMEM/F-12 (GIBCO) supplemented with 1X insulin-transferrin-selenium (GIBCO) and 0.5% Penicillin/Streptomycin (GIBCO). Subsequently, CD34+45- source cells were seeded at confluent density (100,000 cells/cm^2) directly on top of a confluent layer of quiescent SMCs in EC growth medium. The SMC differentiation marker α-SMA was assessedafter 4 days of co-culture. A monoculture of SMCs was used as a negative control and co-culture with mature ECs was used as a positive control.

III. RESULTS

Purity of Isolated CD34+45- Source Cells

Using human umbilical cord blood, CD34+CD45- source cells were successfully isolated with >90% purity in 6 consecutive isolation procedures.

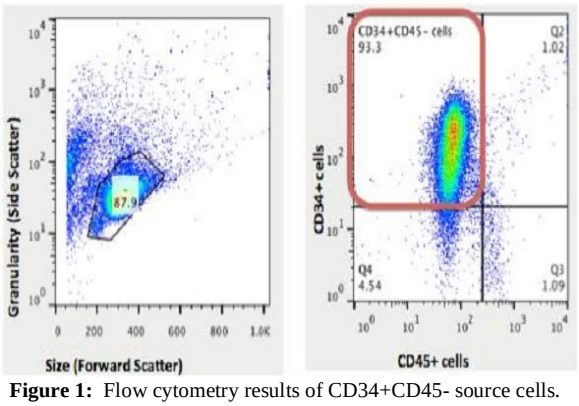

Figure 1: Flow cytometry results of CD34+CD45- source cells.

CD34+45- Source Cells Withstand Fluid Flow Shear Stresses on Uncoated Ti

After seeding of CD34+CD45- cells on Ti for 15 minutes, an average of 39.7± 6.0%, 19.7± 2.1%, 14.7± 6.8% of source cells remained adherent on Ti under laminal fluid flow shear stress of 10, 15, and 20 dynes/cm^2 (n=3 for each shear stress).

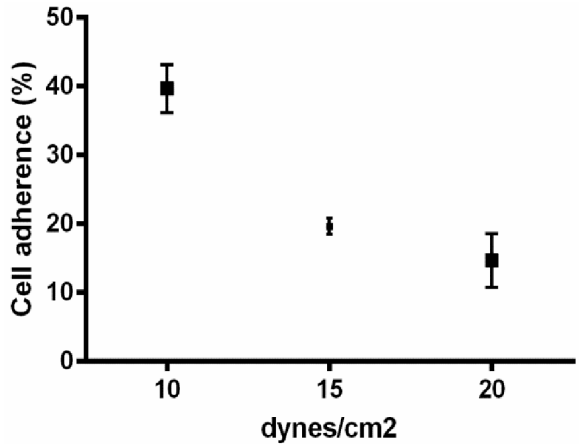

Figure 2. CD34+45- cell adherence on Ti during fluid shear stress following seeding for 15 minutes. Error bar: standard error of the mean.

Differentiation of CD34+45- Source Cells to ECs on Ti

CD34+45- source cells demonstrated EC-like characteristics after culture in EGM-2 medium supplemented by EBM-2 at 37°C for 14 days, as shown by cobblestone morphology (Fig. 3A), DiI-Ac-LDL uptake (Fig. 3B), PECAM-1 positive stain (Fig. 3C) and thrombomodulin expression (Fig. 3D)

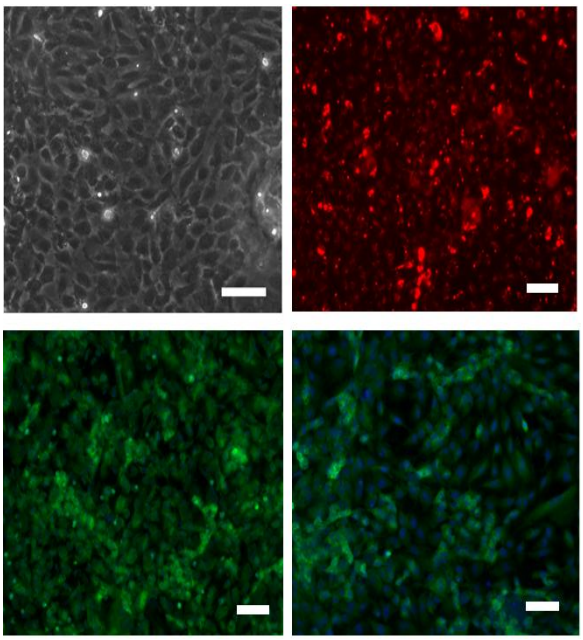

Figure 3. Characterization of differentiated CD34+45- source cells. Phase constrast microscopy exhibiting EC-like cobblestone morphology (A), DiI-Ac-LDL uptake shown in orange (B), PECAM-1 stain shown in green (C) and thrombomodulin expression shown in green (D). Scale bar represents 100 microns.

Platelet Adhesion to Ti Lined with CD34+45- Source Cells

Ti surfaces lined with a confluent monolayer of CD34+45- source cellsafter 14 days in culture (Fig. 4A) were much more resistant to platelet adhesion than bare Ti surfaces (Fig. 4B, red) following a 10 min incubation period. An average of 149.4 ± 178.0 platelets/mm^2 adhered to the cell-lined Ti surfaces, compared to 2372.9 ± 29.2 platelets/mm^2 for the bare Ti surfaces (n=2 slides of each).

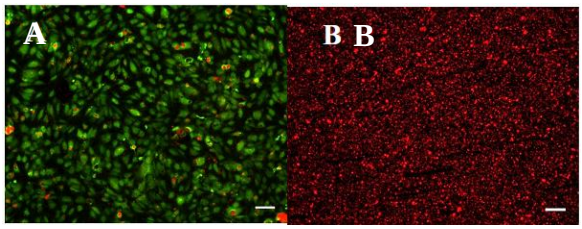

Figure 4. Platelet (red) adhesion to Ti surfaces lined by CD34+CD45- cells (green, A) or to bare Ti surfaces (B).

Inhibition of SMC Dedifferentiation by CD34+45- Source Cells

The expression level of SMC differentiation marker α-SMA in co-culture of SMCs with mature ECs was comparable to the expression level of SMCs in co-culture with CD34+45- source cells, but different from SMCs in monoculture (negative controls). In the negative controls, absence of α-SMA staining indicated dedifferentiation of the SMCs after 14 days (Fig. 5A).The test samples and positive controlswere comparable, indicating that CD34+45- source cells have the potential to maintain SMCsin the differentiated state (Fig. 5B,C).

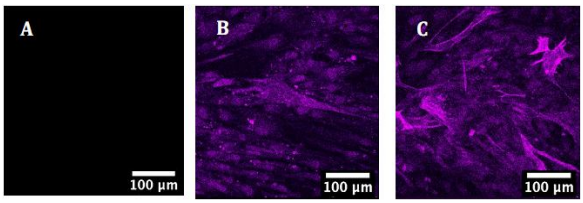

Figure 5. α-SMA expression (magenta) on SMC monoculture as a negative control (A), co-culture of SMCs/ CD34+45- cells (B), and co-culture of SMCs/ECs (C).

DISCUSSION AND CONCLUSION

Our studies demonstrate the feasibility to isolate CD34+45- source cells with >90% purity. The isolated source cells adhered to uncoated Ti surfaces and were able to withstand low fluid flow shear stresses of 10 dynes/cm^2 after 15 minutes static adhesion time, althoughhigher fluid flow shear stresses in the range of 15-20 dynes/cm^2 lead to a lower retention of cells. However, the adhesion may be increased through further optimization of cell seeding, including a longer adhesion time before shear stress exposure and possibly reduced trypsin concentration when harvesting cells prior to seeding Ti.[8]Since CD34+45- source cells have the capacity to adhere to uncoated Ti, it may be feasible to rapidly

seed Ti-coated devices with patients' own source cells in areas of high thrombosis risk (low flow areas) immediately before implantation.[9] The ability of these CD34+45- cells to drastically reduce platelet adhesion to blood-contacting Ti surfaces (Fig. 4) demonstrates one of the potential clinical benefits of this approach because reduction of platelet adhesion would reduce the risk of stent thrombosis.

Further, the source cells differentiated into ECs and showed potential to inhibit SMC dedifferentiation. This finding is important because it addresses one key element of angioplasty (± stenting) failure – the proliferation of SMCs. SMC proliferation and dedifferentiation as well as SMC migration are inter-related events, which propagate intimal hyperplasia and ultimately vessel re-occlusion. If it can be shown that circulating CD34+45- source cells have the potential to inhibit these events and reduce thrombosis, then source cells could be harvested and utilized as an autologous readily available cell therapy product. In addition to being used to coat Ti blood-contacting surfaces, this cell population could also replace mature ECs that are sheared off from the blood vessel intima during angioplasty ± stenting.

A limitation of such a regenerative cell therapy is the low number of circulating CD34+45-source cells. However, methods exist to increase the isolation from patients' blood: It has been shown that the yield of EPCs can be increased by up to 10.2 ± 3.3-fold with prior administration of a mobilizing agent, such as AMD3100 (FDA-approved CXCR4 antagonist Plerixafor),and additionally by up to 100-fold when combined with apheresis, which isolates MNCs from the entire blood volume (5000ml) without loss of red blood cells.[10,11]Following apheresis, CD34+45- source cells may be isolated from patients by positive-/negative-cell selection (e.g. with the FDA-approved CliniMACS system) and then rapidly seeded onto intracranial or peripheral vascular stents with the cell seeding technology developed in our laboratory.[12,13]

Future studies are therefore warranted to investigate the potential of CD34+45- source cells to regenerate damaged endothelium after angioplasty and stenting. The demonstration of CD34+45- source cells' ability to inhibit platelet adhesion and SMC dedifferentiation and proliferation *in vivo*would pave the way for the development of an innovative and personalized therapeutic approach to reduce the risk of in-stent restenosis and thrombosis following angioplasty and stenting.

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