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Biomolecules Based Microarray for Screening Human Endothelial Cells Behavior

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***Abstract*—**Endothelial Progenitor Cell (EPC) based therapies continue to be of interest to treat ischemic events based on their proven role to promote blood vessel formation and thus tissue re-vascularisation. Current strategies for the production of clinical-grade EPCs requires the *in vitro* isolation of EPCs from peripheral blood followed by cell expansion to provide sufficient quantities EPCs for cell therapy. This study aims to examine the use of different biomolecules to significantly improve the current strategy of EPC capture and expansion on collagen type I (Col I). In this study, four different biomolecules were immobilised on a surface and then investigated for their capacity to support EPC capture and proliferation. First, a cell microarray platform was fabricated by coating a glass surface with epoxy functional allyl glycidyl ether plasma polymer (AGEpp) to mediate biomolecule binding. The four candidate biomolecules tested were Col I, collagen type II (Col II), collagen type IV (Col IV) and vascular endothelial growth factor A (VEGF-A), which were arrayed on the epoxy-functionalised surface using a non-contact printer. The surrounding area between the printed biomolecules was passivated with polyethylene glycol-bisamine (A-PEG) to prevent non-specific cell attachment. EPCs were seeded onto the microarray platform and cell numbers quantified after 1 h (to determine capture) and 72 h (to determine proliferation). All of the extracellular matrix (ECM) biomolecules printed demonstrated an ability to capture EPCs within 1 h of cell seeding with Col II exhibiting the highest level of attachment when compared to the other biomolecules. Interestingly, Col IV exhibited the highest increase in EPC expansion after 72 h when compared to Col I, Col II and VEGF-A. These results provide information for significant improvement in the capture and expansion of human EPC for further application.

***Keywords*—**Cardiovascular disease, cell microarray platform, cell therapy, endothelial progenitor cells, high throughput screening.

I. Introduction

VASCULAR disease is one of the main causes of death around the globe [1]. While vascular damage can be somewhat mitigated and controlled through lifestyle modifications, pharmacology and surgery, it remains difficult to re-establish and repair damaged vasculature. Despite this, heart disease remains a leading cause of death and disease burden and further advances to overcome this disease continue to be sought after [2]. To this end, EPCs are well documented to play a critical role in adult vasculogenesis [2]-[4]. In addition, EPC are known to contribute to tissue vascularization in the pathological conditions associated with cardiovascular disease, such as ischemic events in limbs, the retina and the myocardium [4], [5]. Studies show that the sooner that revascularization occurs following an ischemic event, the better the recovery of the injured ischemic organ [5], [6]. EPCs contribute to the formation and repair of blood vessels via a paracrine effect as well as an autocrine effect, wherein they integrate into the arterial wall and secrete proangiogenic factors which promote the proliferation and differentiation within the existing vasculature [5], [7], [8]. In addition, numerous studies have reported EPC-based cell therapies in both animal models and human disease with EPCs re-establishing the integrity of endogenous endothelium, exhibiting regenerative potential [9]-[13]. We contend that identifying suitable biomaterials to support EPC capture and expansion are of real interest with large scale cell expansion required to obtain sufficient cell numbers for cell therapy [14]-[16].

The cell microarray provides a high throughput (HTS) platform that enables the analysis of numerous material-cell interactions using a fraction of the time and analyte materials required for conventional screening processes [17], [18]. This expedites the design and development of advance biomaterials for cell capturing and proliferation [19]. In this study, an HTS platform is created on an epoxy plasma treated glass slide to examine biomaterial candidates to support EPC capture and expansion.

II. Material and Method

Materials

Sterile Dulbecco's phosphate buffered saline (dPBS), antibiotic antimycotic solution, phalloidin-TRITC, Triton X-100, polyethylene glycol-bisamine (A-PEG 3000 kDa), allyl glycidyl ether (AGE) and human Col I, were purchased from Sigma-Aldrich (Australia). Hoechst 33342, trypsin (0.05%, EDTA 0.53 mM, ethanol (absolute, 100%) and acetone (analytical grade, 99.5%) were obtained from ThermoFisher Scientific (Australia). Human vascular endothelial growth factor-165 (hVEGF, 38 kDa) was purchased from Shenandoah Biotechnology (USA).

Ethics Statement

The collection of primary human EPCs from healthy donor peripheral blood was approved by the Human Research Ethics

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Committee of the Royal Adelaide Hospital (RAH), Adelaide, South Australia. Human EPCs were enriched from peripheral blood mononuclear cells being cultured on Col I (from rat tail, Corning, USA) in EBM2 media (Lonza, Australia) + 20% FCS (Hyclone, USA) until colonies formed around days 10-14, based on an established protocol [20]. Cells were then passaged every 3-4 days onto Col I in complete EGM2.

Preparation of EPC Microarray Platform

Prior to deposition of the thin epoxy layer, microscopy glass slides were washed with pirhana solution (mixture of 40% and 60% of hydrogen peroxide (H_2O_2) and sulfuric acid (H_2SO_4) respectively) at room temperature. The slides were washed with Milli-Q water and dried under nitrogen gas. The plasma polymerisation of the AGE monomer was executed as described in our previous paper [21]. Briefly, plasma polymerization was carried out on dried glass slide through continuous wave for 1 min and pulsed wave (DC, duty cycle 1 ms/20 ms) for 2 min where, initial AGE monomer pressure and plasma power were 0.21 mbar and 25 W respectively. Biomolecule solutions including Col I, Col II, Col IV and VEGF were arrayed on AGEpp coated glass slide by non-contact sciFLEXARRAY-ER S3 printing and stored in 4 °C for 16 h. The arrayed samples were passivated with A-PEG as previously described [21]. Afterwards, samples were washed with dPBS and pretreated with 400 U/ml of penicillin, 400 µg/ml of streptomycin, and 1 µg/ml of ampherotericin B (4× antibiotic-antimycotic solution) before cell seeding. Afterwards, EPCs were cultured in EBM 2 supplemented with EGM 2MV bullet kit (Lonza, Australia) and 20% FCS (Corning, USA), were seeded onto biomolecule-arrayed glass slides at a cell density of 6×10^4 for 1 h and 72 h. Samples were stained with 2 µg/ml Hoechst 33342 for 15 min and 100 µM of phalloidin for 30 min at room temperature. Total cell number was counted based on the number of cell nucleis that were stained with Hoechst on each arrayed spot (three different arrayed spots on three different donors).

Surface Characterisation

X-Ray photoelectron spectroscopy (XPS) analysis was performed using a SPECS electron spectrometer on AGEpp coated samples. The pass and resolution energies were 20 eV and 0.5 eV respectively. The amount of chemical component and high resolution spectra were analysed using CasaXPS version 2.3.17 software.

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) analysis was performed using Physical Electronics, Inc. PHI TRIFT V instrument equipped with a $^{79}Au^+$ liquid metal ion gun, operated at 30 kV. A pulsed primary ion beam was used to desorb and ionize species from sample surfaces. Surface analyses were performed using "bunched" Au1 beam setting for surface imaging and spectrometry. Mass axis calibration of the spectra was performed with CH_3^+, $C_2H_5^+$ and $C_3H_7^+$ ions. Experiments were performed in the static mode and under a vacuum of 7.5×10^{-9} Torr.

III. Result and Discussion

Fabrication of Microarray Platform for HTS Screening Experiments

To facilitate protein immobilisation, glass slides were coated with an epoxide plasma polymer (AGEpp) [22]. XPS analysis confirmed the presence of CC/CH and CO functional groups after the AGEpp coating deposition (Table I). The absence of Si signal after coating indicates that the thickness of AGEpp layer is higher than 10 nm (approximate depth of information of the XPS technique). The presence of a C-O peak signal, indicative of the epoxide and the ether components of the AGEpp, further verifies successful coating. Numerous studies have shown that ECM proteins and critical endothelial growth factors such as VEGF play an important role in endothelial cell isolation and expansion [23]-[26], thus, for the purpose of this study, Col I, Col II, Col IV and VEGF were selected. All the selected biomolecules were printed on AGEpp treated glass slides using a printing formulation of concentration of 100 µg/ml and incubated at 4 °C overnight. The arrayed samples were passivated with A-PEG to prevent any non-specific cell and protein attachment between the printed spots [27].

TABLE I
Summary of XPS Elemental Ratios and Component Fitting of C 1s Core Level of Spectra on Cleaned Glass Slide and AGEpp-Coated Glass Slide

% XPS elemental composition				% Contribution in the C 1s fit		
Samples	C	O	Si	C-C/C-H	C-O	O-C-O
Glass slide	20.5	45.2	28.3	-	-	-
AGEpp treated glass slide	78.2	19.6	-	21.5	76.5	1.89

Time-of Flight Secondary Ion Mass Spectrometry (ToF-SIMS)

ToF-SIMS imaging was used to confirm the XPS result. Fig. 1 illustrates positive ion ToF-SIMS images of printed Col I on epoxy functionalised glass slide. The total intensity of the selected positive ion fragments, Na^+, $C_3H_5O^+$ and $C_4H_8N^+$ appeared at the noise level on printed Col I on AGEpp functionalised glass slide, A-PEG passivated functionalised glass slide and printed Col I on AGEpp treated glass slide respectively. Furthermore, $C_4H_8N^+$ and $C_3H_5O^+$ signal traces are strong indicators of homogenous protein and A-PEG coverage.

EPC Microarray on Col I, Col II, Col IV and VEGF for 3 Days

Recent studies have shown that the adsorption of ECM proteins to culture surfaces enhances the expansion rate of EPC [4], [24], [25], [28] and reduces the culture time required to obtain a clinical dose [4]. Therefore, in this study, an EPC cell microarray was constructed to screen immobilised biomolecule for EPC capture and expansion. EPCs were seeded on AGEpp functionalised glass slide with arrays of Col I, Col II, Col IV and VEGF, subsequently passivated with A-PEG. Cell morphology and expansion ratio are illustrated in Figs. 2 (a)-(d). EPC captured on immobilised Col II shows a

significantly ($p < 0.05$) higher cell attachment (~32) after 1 h of cell seeding compared to Col I, Col IV and VEGF. High cell expansion was noted on arrayed Col IV resulting in a 4x increase in cell number ($p < 0.05$) compared to the rest of arrayed biomolecules.

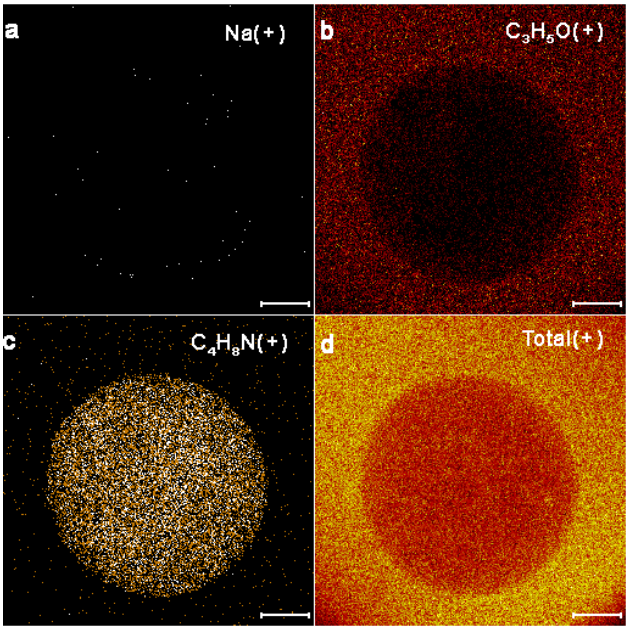

Fig. 1 ToF-SIMS images (500 × 500 µm) acquired on the Col I printed on A-PEG passivated AGEpp functionalized glass slide: Scale bar is 100 µm

IV. Conclusion

EPCs are increasingly being investigated as part of various medical treatments; due to their potential for clinical translation, standard experimental procedures are required for *ex vivo* culture. In this study, we have developed a cell microarray platform on microscopic glass slide to identify possible proteins for surface immobilization in order to support EPC capture and expansion. Protein immobilisation on the array was confirmed using two different surface characterization techniques: XPS and ToF-SIMS. The array demonstrated that the ECM proteins tested (Col I, Col II, and Col IV) could capture and expand EPCs over a three-day period. Our cell microarray results indicate that Col II captures more EPC than other tested biomolecules during the first hour compared to other printed biomolecules, including Col I. Moreover, EPC cells exhibited a significant increase in expansion when seeded on Col IV after 3 days when compared to the current standard operating protocol of seeding early passaged EPC on adsorbed Col I [20]. Based on our results in the microarray format, there is evidence that Col II and Col IV coated surfaces are suitable to sustain EPC isolation and culture.

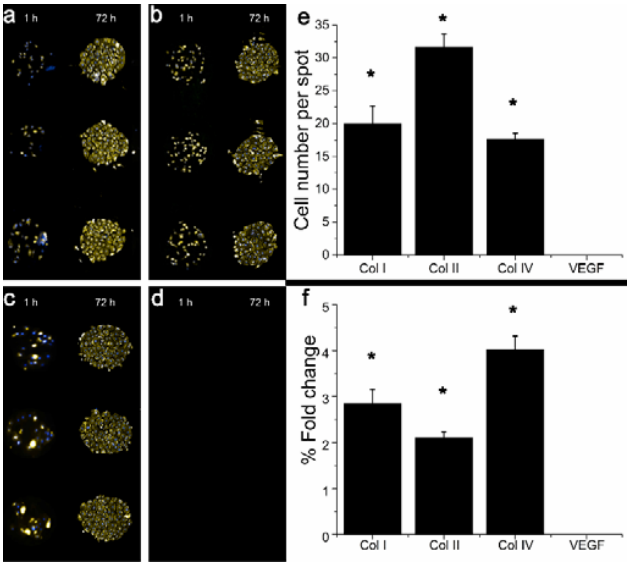

Fig. 2 EPC cell microarray experiment on arrayed Col I, Col II, Col IV and VEGF printed at a concentration of 100 µg/ml on AGEpp functionalised glass slide. (a)-(d) Representative of fluorescence microscopy images of cell adhering to printed Col I (a), Col II (b), Col IV (c) and VEGF (d) after 1 h and 72 h respectively. (e, f) EPC attachment (after 1 h) and expansion (after 72 h) on printed biomolecules. The cell number ratio was obtained from dividing the final cell number at 72 h to cell number at 1 h. Errors bars show standard error (three different spots on three different donors). Scale bar is 100 µm. Statistical significance (*$p<0.05$) was obtained by Student's t-test with Bonferroni correction using the comparison between Col II and Col I and Col IV and Col I

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