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Influence of substrate stiffness on circulating progenitor cell fate

Emanuela S. Fioretta [a,*], Joost O. Fledderus [b,1], Frank P.T. Baaijens [a], Carlijn V.C. Bouten [a]

[a] Soft Tissue Biomechanics and Tissue Engineering, Department of Biomedical Engineering, Eindhoven University of Technology, PO Box 513, 5600 MB Eindhoven, The Netherlands
[b] Department of Nephrology and Hypertension, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

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ABSTRACT

In situ vascular tissue engineering (TE) aims at regenerating vessels using implanted synthetic scaffolds. An envisioned strategy is to capture and differentiate progenitor cells from the bloodstream into the porous scaffold to initiate tissue formation. Among these cells are the endothelial colonies forming cells (ECFCs) that can differentiate into endothelial cells and transdifferentiate into smooth muscle cells under biochemical stimulation. The influence of mechanical stimulation is unknown, but relevant for *in situ* vascular TE because the cells perceive a change in mechanical environment when captured inside the scaffold, where they are shielded from blood flow induced shear stresses. Here we investigate the effects of substrate stiffness as one of the environmental mechanical cues to control ECFC fate within scaffolds. ECFCs were seeded on soft (3.58 ± 0.90 kPa), intermediate (21.59 ± 2.91 kPa), and stiff (93.75 ± 18.36 kPa) fibronectin-coated polyacrylamide gels, as well as on glass controls, and compared to peripheral blood mononuclear cells (PBMC). Cell behavior was analyzed in terms of adhesion (vinculin staining), proliferation (BrdU), phenotype (CD31, αSMA staining, and flow cytometry), and collagen production (col I, III, and IV). While ECFCs adhesion and proliferation increased with substrate stiffness, no change in phenotype was observed. The cells produced no collagen type I, but abundant amounts of collagen type III and IV, albeit in a stiffness-dependent organization. PBMCs did not adhere to the gels, but they did adhere to glass, where they expressed CD31 and collagen type III. Addition mechanical cues, such as cyclic strains, should be studied to further investigate the effect of the mechanical environment on captured circulating cells for *in situ* TE purposes.

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1. Introduction

Mechanoregulation of cell fate has been extensively studied (e.g. Discher et al., 2005; Discher et al., 2009; Ingber, 2002) and substrate stiffness has been recognized as a key factor to regulate cell behavior and, more specifically, stem cells differentiation (Engler et al., 2006). Several research groups confirmed that substrate stiffness can also be used to direct adult cell behavior (e.g. Breuls et al., 2008; Critser and Yoder, 2011) in terms of migration, proliferation, and formation of stress fibers and focal adhesions (Pelham and Wang, 1997; Boonen et al., 2009).

Cell mechanoregulation is of relevance for tissue engineering (TE) approaches, where cells are seeded into a scaffold that replaces the native Extracellular Matrix (ECM). It becomes even more relevant in case of *in situ* TE, where a cell-free scaffold must guide and control cell recruitment, differentiation, and functional tissue formation upon implantation (Mol et al., 2009). Our studies in this area focus on *in situ* vascular TE and aim at capturing and differentiating circulating cells from the blood stream within a scaffold to restore the integrity of small arteries. The blood stream contains several populations of circulating Progenitor Cells (cPCs) that contribute to vascular regeneration (Critser et al., 2011, Richardson and Yoder, 2011). cPCs can adhere to sites of ischemia or vascular injury through a three-stage process (i.e. cell recruitment, rolling, and engraftment) involving $\alpha_4\beta_1$ integrins and cytokines (SDF-1α, VEGF) (Hristov et al., 2003; Chavakis et al., 2008). Moreover, these cells are responsible for *in vivo* endothelialisation of synthetic scaffolds through their differentiation into functional Endothelial Cells (ECs) (Iwai et al., 2004; Yokota et al., 2008). Among the cPCs, Endothelial Colony Forming Cells (ECFCs) have been identified as a candidate cell source for *in situ* vascular TE (Wu et al., 2004). These cells have a high proliferation rate and intrinsic endothelial characteristics (Timmermans et al., 2009). In addition, they can migrate through the elastic lamina of the

Abbreviations: TE, Tissue Engineering; cPC, Circulating Progenitor Cell; EC, Endothelial Cell; ECFC, Endothelial Colony Forming Cell; ECM, Extracellular Matrix; SMC, Smooth Muscle Cell; PBMC, Peripheral Blood Mononuclear Cell; FA, Focal Adhesion; TGF-β1, Transforming Growth Factor β1.

* Correspondence to: Department of Biomedical Engineering, WH 4.12, Eindhoven University of Technology, Den Dolech 2, 5612 AZ Eindhoven, The Netherlands, Tel.: +31 40 247 5415; fax: +31 40 244 3744.

E-mail addresses: e.s.fioretta@tue.nl (E.S. Fioretta), j.o.fledderus@umcutrecht.nl (J.O. Fledderus), f.p.t.baaijens@tue.nl (F.P.T. Baaijens), c.v.c.bouten@tue.nl (C.V.C. Bouten).

[1] Tel.: +31 88 755 9815, fax: +31 30 254 3492.

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vessel wall and transdifferentiate into a synthetic and contractile phenotype similar to smooth muscle cells (SMCs) (Hristov et al., 2003). Therefore, ECFCs have the potential to differentiate in the two different cell lineages (ECs and SMCs) required to regenerate small arteries.

Synthetic scaffolds for *in situ* vascular TE should be able to rapidly recruit desired cell populations to induce tissue formation. Ideally, 3D fibrous and porous scaffolds are used for this purpose to capture cells from the blood stream. Optimization of scaffold properties, being either biochemical or biophysical properties, can be pursued to guide and control cell fate within the scaffold.

Cells captured from the blood stream will perceive a change in mechanical environment. Part of them will adhere to the luminal surface of the scaffold, where they are exposed to blood flow induced shear stresses and the surface properties of the underlying scaffold. The majority of the cells, however, will be captured *inside* the porous scaffold, where they are shielded from the shear stress exerted by the blood. Here, they will be exposed to the mechanical environment of the scaffold, as well as to the local cyclic strains of the vessel wall. In general, synthetic scaffold materials have a higher stiffness (kPa-MPa) compared to native tissues (1–100 kPa) (Engler et al., 2006). Therefore, it is important to identify cell behavior in relation to this parameter.

This study aims to investigate if substrate stiffness has a role in controlling the fate of circulating progenitor cells, and the relevance of this mechanical cue for *in situ* vascular tissue engineering approaches. For this purpose human ECFCs were seeded on polyacrylamide (PA) gels with varying stiffness, and cell behavior was characterized in terms of adhesion, proliferation, differentiation, and collagen production. A combination of αSMA expression and collagen production was used to mark the transdifferentiation potential of ECFCs (Sales et al., 2006). Because ECFCs become adherent after the isolation process, circulating peripheral blood mononuclear cells (PBMC), recruited also on PA gels, were used for comparison.

2. Materials and methods

Reported results were obtained from three independent experiments. In the first two experiments ECFCs were seeded on fibronectin-coated PA gels and glass substrates at day 0. Analyses were performed 2 day (day2) or 4 day (day4) after seeding, with the exception of cell proliferation (determined in the second night after seeding). The third experiment involved the recruitment of PBMCs from a cell solution onto fibronectin-coated PA gels and glass. These cells were only analyzed at day4, because of the low cell yield after 2 day of culture. Material batches, as well as ECFCs batch and passage, were similar for all experiments.

Cell fate was assessed from the following: 1) phenotypic markers against CD31 (differentiation towards EC lineage) and αSMA (transdifferentiation towards SMC lineage) using immunocytochemistry and flow cytometry; 2) cell adhesion and cytoskeletal organization using immunofluorescent stainings for vinculin (focal adhesions, FAs), vimentin (intermediate filaments), and phalloidin (stress fibers); 3) antibodies against collagen type I, III, and IV to identify relevant matrix proteins of the vascular wall and basal lamina.

Table 1
Composition and resulting elastic modulus and thickness of polyacrylamide gels ($n=12$).

Polyacrylamide gels	Soft	Intermediate	Stiff
Acrylamide [% v/v]	5	5	10
Bis-Acrylamide [% v/v]	0.03	0.3	0.26
MiliQ water [% v/v]	75.5	62.1	51.15
Elastic modulus [kPa]	3.58 ± 0.90	21.59 ± 2.91	93.75 ± 18.36
Thickness [mm]	0.26 ± 0.07	0.18 ± 0.03	0.23 ± 0.07

2.1. Substrate preparation and characterization

PA gels were prepared to obtain three different stiffness values within the range of physiological tissue stiffness (1–100 kPa; Discher et al., 2009). The gels were prepared using a method adapted from Pelham and Wang (1997). N,N′,N′-methylene-bis-acrylamide (Sigma-Aldrich) was mixed with Acrylamide (Sigma-Aldrich), 50 mM HEPES (10% v/v, Sigma), miliQ water, and crosslinked using 10% ammonium persulfate (APS, 1/200 vol/vol; Fisher, Pittsburgh, USA) and N,N,N′,N′-tetramethylethylenediamine (TEMED, 1/2000 vol/vol; Merck). Droplets of the acryl–bisacrylamide solution were casted on a aminosilanized (using (3-aminopropyl)trimethoxysilane, Sigma-Aldrich) glass coverslip (Menzel) and covered with another glass coverslip, siliconized with 50% SurfaSil solution (Thermoscientific) in acetone. After removing the top coverslip, a 50 μg/ml coating of fibronectin from human plasma (Sigma-Aldrich) was crosslinked to the gel surface using heterobifunctional sulphosuccinimidyl-6-(4′-azido-2′-nitrophenylamino)-hexanoate (Sulfo-SANPAH, Pierce Biotechnology). Fibronectin, and not collagen, was chosen as a coating of the gels because of its know adhesive properties for ECs and to avoid cross-talk with autologous production of collagen by the cells (see below). Gels were sterilized under UV light for 30 min and incubated at 37 °C in medium, prior to cell seeding. Glass coverslips, coated with the same fibronectin solution, were used as controls. These were sterilized overnight with 70% ethanol. Gel concentrations used, elastic moduli, and thicknesses of the gels obtained are reported in Table 1. A staining (Table 2) of the fibronecting coating was performed to verify homogeneous coating distribution on the different substrates.

The elastic modulus (E) of the PA gels was determined by indentation tests, as described by Boonen et al. (2009). Local indentation was applied to the center of the gels using a spherical indenter (Ø 2 mm), while measuring indentation force and depth. Afterwards, a numerical model was iteratively fitted to the experimental data using a parameter estimation algorithm (Cox et al., 2008). The mechanical properties of the gels were determined for each separate experiment, and averaged (mean ± sd) using four gels for each stiffness group.

2.2. Cell isolation and culture

PBMCs were isolated from human peripheral blood (fresh buffy coat from a single healthy donor, obtained from Sanquin Blood Supply Foundation, Nijmegen, the Netherlands) through a gradient centrifuging method using Ficoll–Paque Premium (GE Healthcare). Cells were preserved in liquid nitrogen until use. To isolate ECFCs, PBMCs were seeded onto collagen-coated (10 μg/ml) culture plates, as previously described (Lin et al., 2000). Cells were cultured in EGM-2 medium: EBM-2 medium (Lonza) with 10% fetal bovine serum (FBS, Greiner Bio-One), 100,000 IU/l penicillin and 100 mg/l streptomycin (Lonza), 4 mM L-Glutamine (Lonza), and supplemented with EGM-2 Single Quots (Lonza). After 24 day of culture, ECFC colonies with characteristic cobblestone morphology could be detected. These cells were then harvested, expanded, and preserved in liquid nitrogen until further use.

Prior to the experiments, ECFCs were thawed and cultured in EGM-2 medium, which was changed every other day, until 90% confluence was reached. Cells were then harvested using 0.05% trypsin-EDTA and 3000 cells/cm^2 were seeded onto the PA gels. Circulating PBMCs were seeded on the gels immediately after thawing, using a suspension of physiological cell density (10^6 cells/ml of medium).

Table 2
Primary and secondary antibodies used for immunocytochemistry of different markers.

Marker	Primary antibody	Secondary antibody
CD31	Mouse anti-human IgG1; 1:100 v/v (Dako)	Alexa fluor 555 goat anti-mouse IgG; 1:300 v/v (Invitrogen)
Collagen type I	Mouse anti-human IgG1; 1:100 v/v (Abcam)	Alexa fluor 488 goat anti-mouse IgG1; 1:200 v/v (Invitrogen)
Collagen type III	Rabbit anti-human IgG; 1:200 v/v (Abcam)	Alexa fluor 555 donkey anti-rabbit IgG(H+L); 1:300 v/v (Invitrogen)
Collagen type IV	Mouse anti-human IgG1; 1:100 v/v (Abcam)	Alexa fluor 555 goat anti-mouse IgG1; 1:300 v/v (Invitrogen)
Fibronectin	Rabbit polyclonal IgG; 1:400 v/v (Sigma)	Alexa fluor 488 donkey anti-rabbit IgG; 1:300 v/v (Invitrogen)
Phalloidin	Phalloidin Fluorescein Isothiocyanate (Sigma)	labeled FITC; 50 μg/ml labeled TRITC; 50 μg/ml
Vimentin	Mouse anti-human IgM; 1:1000 v/v (Abcam)	Alexa fluor 488 goat anti-mouse IgM; 1:200 v/v (Invitrogen)
Vinculin	Mouse anti-human IgG1; 1:400 v/v (Sigma)	Alexa Fluor 488 goat anti-mouse IgG1; 1:300 v/v (Invitrogen)
αSMA	Mouse anti-human IgG2a; 1:500 v/v (Sigma)	Alexa Fluor 488 goat anti-mouse IgG2a; 1:300 v/v (Invitrogen)

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