Substrate Stiffness Modulates Endothelial Phenotypic Transition

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Statement of Purpose: Atherosclerosis, which is characterized by progressive stiffening of the arteries and formation of occlusive plaques, accounts for more than 15% of deaths worldwide. The most common treatments for atherosclerotic vessels are bypass grafting and stenting. However, about 40% of the vascular grafts fail within 2 years of implantation, mainly due to graft stenosis. Recent studies demonstrated the incidence of endothelial-to-mesenchymal transition (EndoMT) during graft stenosis progression. Despite evidence of the correlation between mechanical properties and EndoMT progression, the regulatory effect of substrate stiffness on progression of EndoMT remains poorly defined. The aim of this study is to investigate the effect of mechanical properties of the underlying matrix on progression of EndoMT and its probable mediatory effect on transforming growth factor-β (TGF-β) induced EndoMT, and to reveal the mechanism underlying this process. We hypothesize that substrate stiffness modulates vascular endothelial cell (EC) phenotype and regulates the stimulatory effect of TGF-β, the known inflammatory cytokine, on progression of EndoMT.

Methods: Human aortic ECs (HAECs) were cultured on polyacrylamide hydrogel-coated plates (Matrigen) with stiffnesses of 4 kPa or 100 kPa. Standard tissue culture plates (TC, 100,000 kPa) were used as the control. The expression of a panel of mesenchymal and EndoMT marker genes including SNAIL, SM22, Calponin, Twist, and α-SMA was investigated using RT-PCR. The gene expression studies were performed for HAECs with/without exposure to the known EndoMT stimulator, TGF-β (20ng/ml), at time points up to 14 days. The gene expression of the cells seeded on TC at the day of starting TGF-β treatment was used as the reference. The EndoMT progression was further studied at protein level by immunofluorescence staining of mesenchymal (SM22) and EndoMT (SNAIL) protein markers at day 14 of the culture/treatment. The cells were also stained with phalloidin to characterize the morphological changes in transitioning HAECs in response to substrate stiffness and TGF-β treatment.

Results: Gene expression studies using RT-PCR demonstrated that at day 6 of cell culture on substrates of varying stiffness with/without TGF-β treatment, HAECs expressed mesenchymal (SM22, calponin, Twist, α-SMA) and EndoMT (SNAIL) markers in a stiffness-dependent manner, where stiffer substrates induced more phenotypic transition. Interestingly, in the presence of TGF-β, a known stimulator of EndoMT, the cell culture on the hydrogels of physiological stiffness could partially abrogate the expression of mesenchymal markers compared to TC. The EndoMT progression was further studied at protein level by immunofluorescence staining of mesenchymal (SM22) and EndoMT (SNAIL) protein markers. The results showed that with increasing the substrate stiffness, SM22 protein expression was notably increased, and the TGF-β-induced SM22 expression was significantly inhibited on substrates of lower stiffness. Quantitative analysis of the expression of SNAIL demonstrated that HAECs seeded on the hydrogels expressed lower levels of the protein compared to the cells seeded on TC (**Figure 1**). The cell size measurements using phalloidin stained HAECs also indicated that by increasing the stiffness the mean size of the cells was increased from 408 to 583 μm^2 without TGF-β treatment and from 485 to 905 μm^2 in presence of TGF-β, and the same trend was observed for the size distribution (heterogenicity) of the cells. This demonstrates the mediatory effect of both substrate stiffness and TGF-β treatment on the morphology of the cells, resulting in a more heterogeneous cellular population on the stiffer substrate under TGF-β treatment.

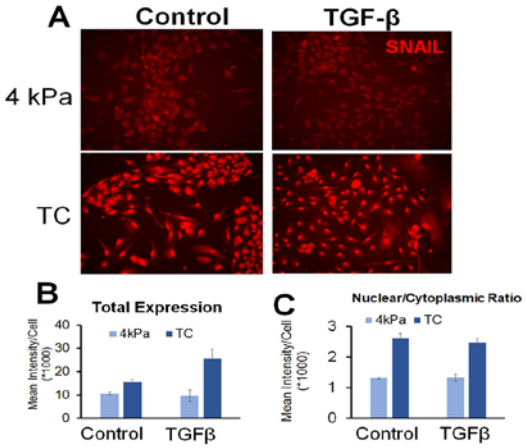

Figure 1. SNAIL expression and localization on hydrogels (4 kPa), in comparison to tissue culture (TC) after 14 days.

Conclusions: We have demonstrated that substrate stiffness effectively mediates progression of EndoMT. The HAECs cultured on the stiffer substrates expressed markedly higher mesenchymal markers at gene and protein level. Furthermore, the morphology and heterogenicity of the cellular population showed to be influenced by rigidity of the substrate. These findings provide the basis for future preventative strategies and may also facilitate development of anti-stenotic vascular grafts with proper material design and long-term patency for treatment of arterial diseases.

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