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LYMPHOCYTE NANOMECHANICS IN RESPONSE TO STENT IMPLANTATION

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INTRODUCTION

Changes in the arterial mechanical environment due to stent implantation are the main causes of the initiation of thrombosis and restenosis [1]. The mechanical properties of live cells can affect their physical interactions with the surrounding extracellular matrix, potentially influencing the process of mechanical signal transduction in living tissues. Previous studies have described the infiltration of inflammatory cells and immunocytes (T-lymphocytes) in restenotic tissue. This inflammatory response is characterized by the activation of circulating leukocytes that express adhesion molecules on the cell surface [2]. In this work we investigate stent-induced alterations in the nanostructure, cytoskeleton and mechanical properties of circulating lymphocytes in an effort to establish mechanical biomarkers to access stent-induced inflammation in the adjacent vascular tissue.

METHODS

Animal stenting: Male atherosclerotic ApoE−/− (30±5 g) and male CD1 (40±5 g) mice were used throughout the study (Project license: CY/EXP/PR.L9/2019). Custom made self-expanding nitinol stents (0.7 x 3.3 mm, closed-cell design with a diamond-shaped pattern; Admedes GmbH, Pforzheim, Germany), were implanted following the experimental protocol reported by Simsekyilmaz et al. 2013 [3] which describes a rapid and accessible procedure of stent implantation in mouse carotid artery.

Lymphocyte isolation: Whole blood (0.5-1 ml per animal) was collected from control (non-stented; n=3 per strain) and stented (12 weeks post implantation; n=3 per strain) mice, by direct cardiac puncture using a heparinized syringe. Mononuclear cells (MNCs) were isolated from whole blood using the SepMate™ procedure and highly purified CD4+ T lymphocyte subsets were enriched via the RosetteSep™ protocol (Stemcell Technologies, Vancouver, BC, CA).

Cell culture: Isolated lymphocytes were cultured in RPMI1640 medium, supplemented with penicillin 100 IU/ml, streptomycin 100 mg/ml, L-glutamine and 10% newborn calf serum at 37 8C in a humidified atmosphere of 5% CO2. The cells were cultured either on 35 petri dishes or glass cover slips, both coated for 5 minutes with Poly-L-lysine hydrobromide (Sigma).

Atomic Force Microscopy (AFM): AFM characterization was performed to probe the mechanical properties of live cells using a Molecular Imaging-Agilent PicoPlus AFM system and V-shaped soft silicon nitride probes (MLCT-Bio, probe C, Bruker). Petri dishes with the cultured cells, either for 2h (same day group) or for 24h (next day group), were directly mounded on AFM sample plates. In an area of 1x1μm near the center of the cells, 8x8 points of force curves were collected and analyzed by the freeware software AtomicJ so as to calculate the sample's Young's modulus using the Hertz model (for cells a 0.5 Poisson ratio was used). All mechanical properties measurements were recorded with a set point of 1nN normal force. For the mechanical characterization at least 30 live cells per condition from 3 independent experiments were studied, while attention was paid to always perform the measurements in less than 40min per experiment. Imaging of fixed cells (20 min with 4% paraformaldehyde-PFA) was performed in tapping mode with silicon probes (ACT-Applied Nanostructures). The AFM image processing was performed by using the PicoView software (Agilent) and the freeware scanning probe microscopy software WSxM.

Cell Immunostaining: Cells were first fixed with 4% PFA for 20 min and then a permeabilization buffer containing phosphate buffered saline, 2mg/ml Bovine Serum Albumin, and 0.1% Triton X-100. Then, cells were incubated with phalloidin for 1 hour at room temperature. Finally, cells were washed again three times with the permeabilization buffer and incubated for 2 min with 4',6-Diamidino-2-Phenylindole

(DAPI, Sigma). All coverslips were then mounted on a slide and observed under an Olympus BX53 fluorescent microscope equipped with an Olympus XM10 Monochrome CCD camera.

Stress fibers: For the characterization of the actin stress fibers, the FilamentSensor tool was used. In the reconstructed images each color corresponds to a different fiber orientation. Stress fiber orientation was assessed using the order parameter S=cos2θ, where he higher the value of S is, the more oriented the fibers become.

Cells' shape: Cell elongation was assessed using optical microscopy images. ImageJ software was used to automatically measure factor E from cells. Factor E equals to the long axis divided by the short axis minus one. Thus, E is zero for a circle, and one for an ellipse with an axis ratio 1:2. The cells that presented E values 0–0.5 were considered as spherical, 0.5–1 as ellipsoid, and E values higher than 1 as elongated. We used the optical microscope images from the cells so as to measure the cells circularity.

RESULTS

Firstly, we studied normal lymphocyte morphology. Our data demonstrated that lymphocytes from non-stented (control) ApoE and CD1 mice present typical spherical shape (Fig.1A), while high resolution AFM images showed cellular microvilli at the cells' surface and pseudopodia at the edge of the cell (Fig.1B). Furthermore, stress fibers where mainly located at the periphery of the cells, without any significant orientation pattern (Fig. 1A).

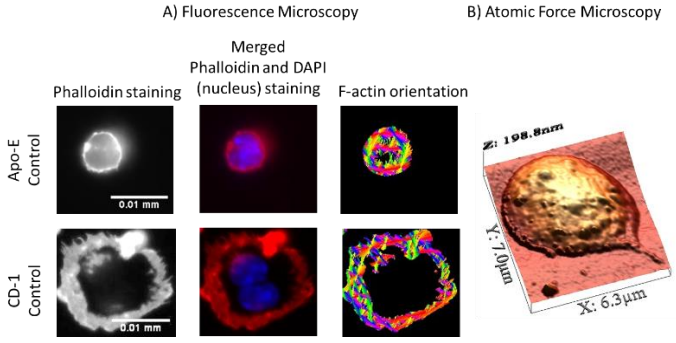

Figure 1: Representative images of lymphocytes (from control ApoE and CD1 mice) via A) fluorescence and B) AFM microscopy.

AFM studies on live cells from stented mice, highlighted that lymphocytes exhibit a significantly higher Young's modulus value. This pattern was observed in cells from both mouse strains, and in lymphocytes that were studied *in vitro* either 2h (same day) or 24h (next day) after isolation (Fig.2).

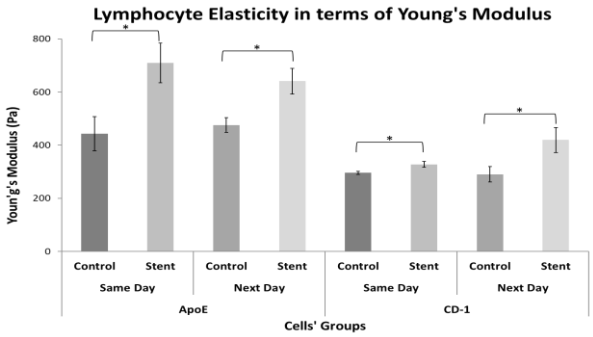

Figure 2: Young's modulus of lymphocytes (2h and 24h after isolation) from control vs stented mice. (* indicates p<0.05)

Subsequently, we used fluorescent microscopy to study whether inflammation alters lymphocytes morphology and F-actin stress fibers characteristics, which are considered to be the major cytoskeleton characteristic responsible for cells' mechanical properties. The results show that lymphocytes morphology was altered, as lymphocytes were becoming more elongated (Fig. 3 A (left) and B). Furthermore, notable cytoskeleton changes were observed, including F-actin stress fibers distribution and orientation (Fig. 3 A (right) and C). Stress fibers were distributed throughout the cell body, and more elongated patterns were formed. We believe that these alterations in F-actin fibers are due to the activation of lymphocytes as a result of the stent-induced inflammatory response. Consequently, cellular remodeling significantly modified the cell's mechanical properties, as assessed with AFM.

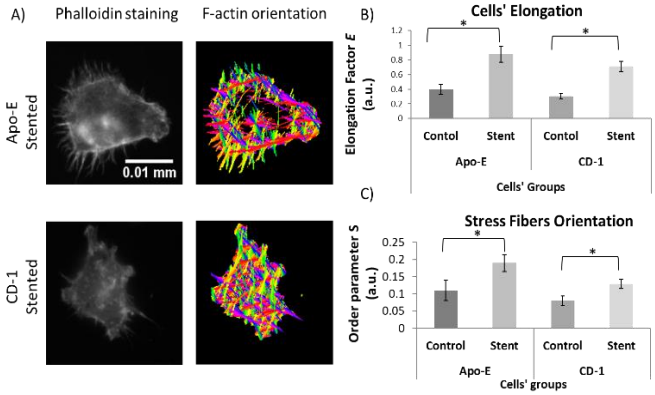

Figure 3: A) Representative images of lymphocytes (from stented ApoE and CD1 mice). Quantification of cell's B) elongation and C) stress fibers orientation. (* indicates p<0.05)

DISCUSSION

Restenosis appears to be associated to inflammatory and immunological activity that persists several months after coronary intervention. AFM has emerged as a powerful tool for studying important dynamic cellular processes in real time and it has been recently demonstrated that it can be used for developing nanomechanical biomarkers from single cells up to tissue samples [4].

Our research results demonstrated that lymphocytes from stented mice present an increase in their stiffness. Cell remodeling was observed in two different mice strains, the most widely used model of atherosclerosis, the ApoE-/- (for which changes due to lymphocyte activation are more profound) and the multipurpose CD1 strain. It is noted that the elevated Young's modulus is accompanied with the cytoskeleton rearrangement. Our findings suggest that the nanomechanical properties of circulating lymphocytes, could potentially be used as a biomarker to access stent-induced inflammation and possibly early stages of in-stent restenosis.

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