Investigation on the effects of variable shear stress on monocyte cell morphology

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Microchannels fabricated by soft lithography have been favourable in mechanobiological applications for cell culture platforms, especially shear stress studies. Normally, physiological shear stress levels for veins are between 10 and 70 dynes/cm^2 (1–7 Pa) and arteries between 1 and 6 dynes/cm^2 (0.1–0.7 Pa). In this work, a circular system has been set up by microfluidic pump to mimic physiological environment of monocyte cells for monitoring structural changes at various levels of shear stresses. Shear stresses at 4, 15 and 45 Pa, low, medium and high stresses, respectively, simulated by CFD have been applied on THP-1 cells under different flow rates for 0.5 and 3 h. These cells, which passed through microchannels under these experimental conditions were analysed for their F-actin content by staining with phalloidin and determination of mean fluorescence intensity by fluorescence microscopy and flow cytometry. An increase in THP-1 monocytic cell actin polymerisation is significant in the case of 15 Pa, and slight in the case of 45 Pa shear stress conditions for 0.5 h. This may reflect the structural adaptation of monocytes during a pathological condition such as atherosclerosis.

1. Introduction: The study of cell responses to mechanical stimulations which exist in the body is crucial within the context of the function of tissues and organs. The process is defined as mechanotransduction, where cells sense mechanical stimulations such as intrinsic and extrinsic ones and then convert them into biochemical responses [1, 2]. In addition to that, the cellular mechanotransduction process is essential to maintain physiological development of cell and tissue. It was shown that processes such as protein synthesis, secretion, adhesion, growth, development, migration and apoptosis are modulated by mechanotransduction on the cellular level [3–6].

The functional pathway of the mechanical forces which cells are subjected in their environment must be revealed to understand how individual cells sense and respond to them. The mechanical forces applied to individual tissue will be transmitted to cells by specialised sensing elements. For example, receptors and ion channels on the cell membrane are involved in sensing of physical cues [7, 8]. Furthermore, cells also respond to mechanical forces through external or internal stimulations such as membrane tension, rigidity of extracellular matrix, shear stress, hydrostatic pressure and compression. Those mechanical forces have effects on gene expression as well as size and shape of the cells [9, 10]. For instance, differentiation of mesenchymal stem cells into different cell types depends on the matrix stiffness that influences focal-adhesion structure and cytoskeleton [11]. Another example is blood vessels that remodel themselves in response to changes in blood pressure and shear stress [12] in early and late stages depending on exposure time [13].

Although parallel-plate fluid flow chamber has been used widely to mimic in vivo fluid flow of cell types such as endothelial cells (ECs) [14], osteoblasts [15], and stem cells (Mesenchymal stem cells (MSCs)) [16]. However, due to some limitations of non-uniform and uncontrolled flow, there should be a microfluidic platform in order to study mechanical effects (shear forces) on cultured cells in a comprehensive way [17]. Microfabricated systems have been attractive for use in shear stress studies because of providing controllable and reproducible environment for cultured cells. Microfluidic devices can be integrated with cell culture that have several advantages compared with standard cell culture platforms, such as consuming small amounts of medium and reagents, fast analysis time and low cost [18–20]. Poly(dimethylsiloxane) (PDMS) is widely used for fabrication of microfluidic devices in soft lithography process that has features compatible with biological systems such as optical transparency, gas permeability, low toxicity and flexible surface chemistry [21]. Microfluidic-based systems allow analysis of systems from single cells to complex tissues by modelling microstructure considering differences [22].

The type of interstitial, lymphatic or circulatory shear stresses modulates physiological processes of cells, such as growth, functioning, development, alignment and apoptosis. Therefore, it is crucial to reveal the shear stress on activities in the cells. Recently, fluid flow in circulatory system has been studied widely in vitro with ECs [23] and in vivo with animal models [24]. Adhesion behaviour between monocytes and ECs has been also studied to understand its quality under the shear stress. However, there has not been any designed specific platform to mimic vessels in terms of examining the effects of shear stress on monocyte cells as they are in circulatory system. It is known that migration of leukocytes in blood which has a high flow stress requires the dynamic reorganisation of actomyosin system [25, 26]. To date, no studies on the effect of shear stress on changes in the cytoskeletal structure of monocytes have been reported. Therefore, we aim to investigate the effect of shear stress on F-actin content in monocytes. For this purpose, in this Letter, PDMS cavitated microchannels were designed to create shear stress and pressure points on monocytes. A circular microfluidic system was used to mimic physiological environment of monocyte cells in order to monitor structural changes at high level shear stress. Shear stresses at various values including 4, 15 and 45 Pa were applied on THP-1 cells during 0.5 and 3 h, time points. The changes in cytoskeletal assembly in monocytes were determined by analysing levels of filamentous actin (F-actin) by both microscopy and flow cytometry.

2. Materials and methods

2.1. Fabrication of microfluidic channels: Microfluidic channels were fabricated by soft-lithography techniques as follows: master template fabrication, PDMS replica moulding and plasma oxidation. Making master template required a negative photoresist SU-8 3050 (MicroChem Corp.) deposited on Si-wafer (University Wafer) and distributed by spinner. After the spin coating step, the Si-wafer with SU-8 was put on hot plate at 95°C for 40 min. Then Si-wafer with SU-8 was processed by lithography under

UV light. Si-wafer was put on the hot plate in post exposure bake step. SU-8 on Si-wafer was exposed to developer (1-methoxy-2-propanol acetate) for dissolving the soluble parts for 15 min and then isopropyl alcohol and deionised water, dried with nitrogen. After all processes, the hard bake process was performed at least 2 h. Aluminium foil was coated onto petri dish to avoid leaking PDMS Sylgard 184 (Dow Corning) onto the dish and then the Si-wafer with the SU-8 master was located onto dish. PDMS and the curing agent were mixed at 10:1 ratio. The mixture was poured onto SU-8 master template and air bubbles were removed under vacuum by oven. Finally, the bubble free PDMS was put into oven for solidification at 75°C for 2 h. At the end, PDMS and glass slides were exposed to plasma oxidation for providing surface modification in the following day. They were put into the plasma device on a glass plate and exposed to medium Radio frequency (RF) level O_2 plasma for 40 s [10].

2.2. Design of microfluidic channels: Microfluidic channels used in these experiments were designed to have cavities for creating variable shear stress and pressure differences. Depending on the expected shear stress level, microchannel size and geometry can be modified by soft lithography [27]. Channel dimensions are designed different from each other for creating different shear stress values and pressure points that is represented in Fig. 1.

2.3. Experimental setup: A circular system set up has been represented schematically in Fig. 2. ExiGo Pump (Cellix Ltd.) provide cell circulation set up by refilling property when medium volume in syringe is injected into microfluidic channel by pumping. The syringe in the pump is connected to microfluidic valve through tubing (Elveflow) and this microfluidic valve is connected to microfluidic sensor. The tubing on the output of this microfluidic sensor is connected to microfluidic channel. The tubing on the outlet of the microfluidic channel is placed to source falcon tube. The pump refills the syringe with cells in the medium from this falcon tube.

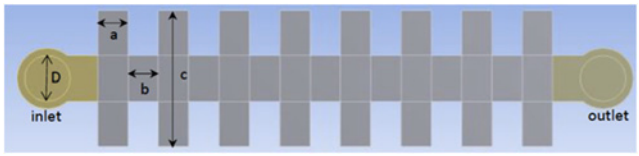

Fig. 1 *Microfluidic channels used in the experiments for channel 1, a = b = 500 µm; c = 2250 (=3 × 750 or ER = 3) µm; D = 750 µm and channel 2, a = b = 1000 µm; c = 3000 (=3 × 1000 or ER = 3) µm; D = 750 µm. Total length of this channel from inlet centre to outlet centre is 9.161 mm, height is 100 µm and has more cavities than this represented figure*

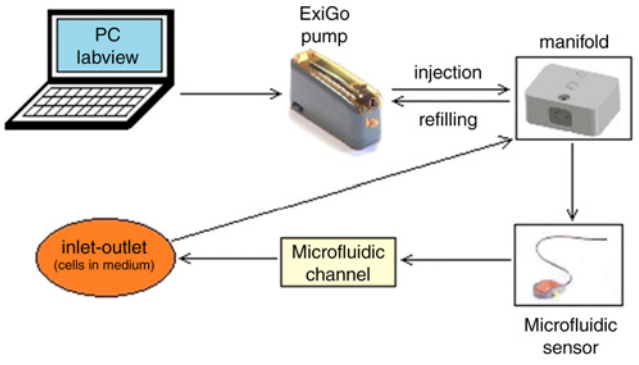

Fig. 2 *Schematic representation of circular set up of experiments performing with THP-1 cells*

2.4. Cell culture: THP-1, human monocytic cell line was a gift from Nesrin Ozoren, Bogazici University, Turkey. THP-1 cells were maintained at 37°C and 5% CO_2 environment in complete RPMI 1640 medium (Gibco) supplemented with 10% Fetal bovine serum (FBS) (Biowest) and 1% penicillin–streptomycin (Lonza). Cultures were maintained by addition of fresh medium or replacement of medium after centrifugation at 160*g* for 5 min. Cell concentration was not allowed to exceed 1×10^6 cells/ml. Experiments were performed with 10 ml cell suspension at 0.8×10^6/ml cell density. Static control cells were incubated in cell culture flasks at the same density throughout the experiment [28].

2.5. Fluorescence staining: THP-1 cells were obtained by centrifugation and were fixed in 4% paraformaldehyde in Phosphate-buffered saline (PBS) (Biochrom) for 10 min at room temperature. Then, cells were washed with PBS solution containing 2% FBS and permeabilised in NH_4Cl (Sigma) buffer with 0.1% saponin (Sigma). THP-1 cells were stained with phalloidin – Tetramethylrhodamine (TRITC) (1:200 dilution; Sigma) in PBS solution supplemented with 0.1% saponin for 20 min at room temperature. After washing, stained cells were mounted onto glass slides and imaged with a fluorescence microscope (Zeiss Axiovert A.1 inverted fluorescence microscope) with a 40× objective. Mean fluorescence intensities (MFIs) were analysed by ImageJ 1.51 software.

2.6. Flow cytometric determination of F-actin in monocytes: THP-1 cells were obtained by centrifugation and were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. Then, cells were washed with PBS solution containing 2% FBS and resuspended in permeabilisation buffer (BioLegend) for 10 min. Intracellular F-actin was stained by incubation of fluorescein isothiocyanate (FITC) – phalloidin (Santa Cruz) at 1:50 dilution in permeabilisation buffer for 1 h. After washing, MFI of stained cells was measured by flow cytometry using BD Accuri C6 Flow Cytometer (Becton Dickinson). The analysis of MFI values was performed by FlowJo software. By using forward angle light scatter and side angle light scatter, cell debris were excluded from analysis by gating of monocytes. The cell counts that each analysis was performed were identical for each group.

2.7. Statistical analysis: All statistical analyses were performed using GraphPad prism software. Values are quoted as mean ± SEM. Differences between the shear stress treated groups and control group were analysed by analysis of variance (ANOVA) test.

3. Results

3.1. Simulation by computational fluid dynamics (CFD): Shear stress is defined as tangential force of the flowing blood that affects ECs lining on blood vessels and other blood cells such as immune cells by direct action with flow or indirect action with transporting chemicals and similar compounds [29–34].

Normal physiological shear stress ranges from 10 to 70 dynes/cm^2 (1–7 Pa) through arteries, whereas shear stress ranges from 1 to 6 dynes/cm^2 (0.1–0.6 Pa) through veins in the body [35–37]. Shear stresses applied on THP-1 cells in this study were 4, 15 and 45 Pa that were simulated by ANSYS-Fluent Flow Analyser. 4 Pa shear stress was created to apply on cells using channel-1 under 2400 µl/min, 15 Pa shear stress was created using channel-2 under 1200 µl/min and also, 45 Pa shear stress was created using channel-2 under 3600 µl/min by simulation studies. Shear stress levels were calculated and meshing was analysed at higher flow rates. It was seen that the results were found to be independent of the mesh. CFD analysis was performed under stable input mass flow rate and output pressure and the pressure difference between input and output sections were obtained. In time independent analysis, mass flow inlet and pressure outlet were determined as boundary conditions and no-slip condition was

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applied on the wall and the system was accepted as isothermal. Analysis was performed by Fluent software as 3D, constant and incompressible. Water was used as liquid.

3.2. 15 Pa shear stress increases the intensity of F-actin: It was important to consider whether there would be any changes in cytoskeleton because of subjecting cells into different shear stress levels. Actin is defined as mechanosensitive element in terms of characterising cell response in microfluidic systems [38]. It was reported that shear stress as a biomechanical force acts on adherent cells such as ECs by regulating actin cytoskeleton remodelling. Also, researchers targeted to probe cytoskeleton elements such as tubulin and actin by contraction forces apart from shear stress [39]. However, there is no clear information regarding changes in actin cytoskeleton of monocyte cells when subjected to shear stress. Therefore, we investigated the content of F-actin in monocytes under various shear stresses by microscopy and flow cytometry.

In this Letter, 4, 15 and 45 Pa shear stress levels during 0.5 and 3 h were applied on THP-1 cell line. We selected 4, 15 and 45 Pa as low, medium and high shear stress, respectively. THP-1 cells, which were not subjected to a flow, were kept as static cell group. The F-actin was labelled with phalloidin in both static and shear stress induced experimental groups of THP-1 cells and the cell content in F-actin was determined by fluorescence microscopy and subsequent semi-quantitative analysis of MFI with Image J 1.51 software. Even though we did not detect a change in cell shape or F-actin distribution of shear stress applied THP-1 cells, a significant increase in the content of F-actin was determined in all experimental groups (Figs. 3*a* and *c*). The increase in the

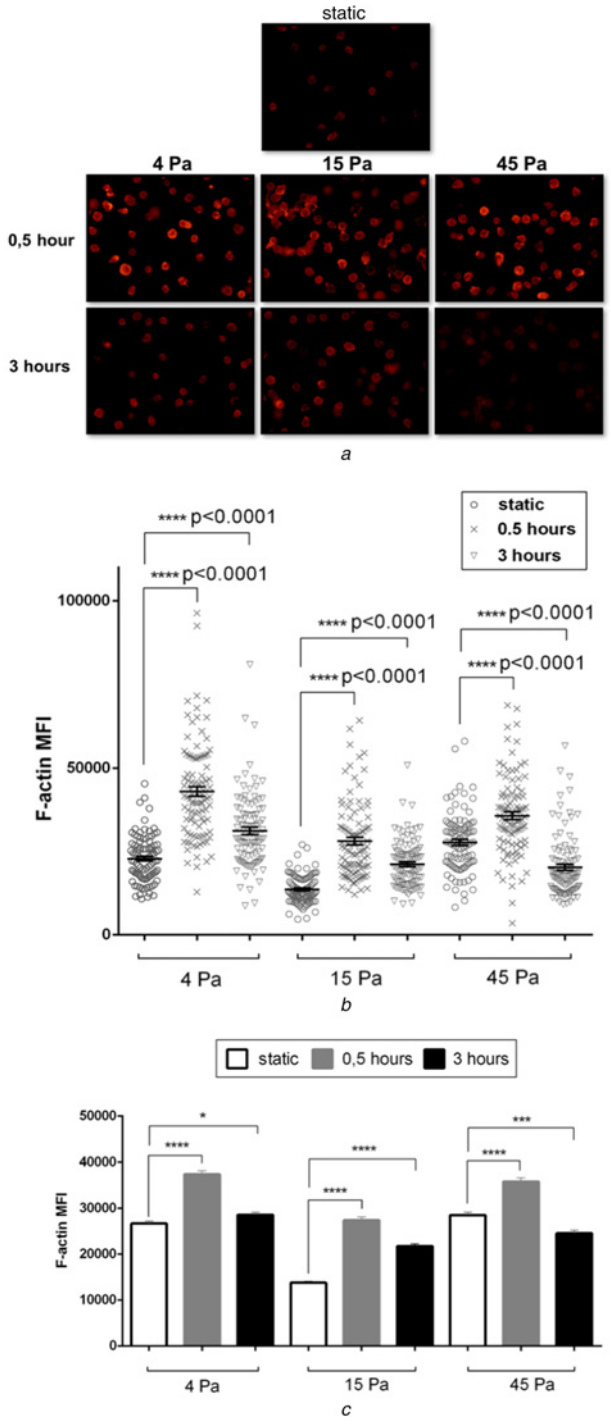

Fig. 3 *Effect of shear stress on F-actin content in THP-1 monocytes analysed by fluorescence microscope. THP-1 cells were kept under static or various shear stress conditions (4, 15 and 45 Pa) for 0.5 and 3 h (n = 2 per treatment group; two independent experiments). Cells were fixed, permeabilised and stained with phalloidin – TRITC (red) and examined with fluorescence microscopy*

a Representative micrographs of F-actin stained THP-1 cells under static or various shear stresses. The magnification: 40×

b For each experimental condition, the MFI of 100 cells was determined

c Each value represents the mean of MFI of two independent experiments. *p* values determined by ANOVA (±SEM)

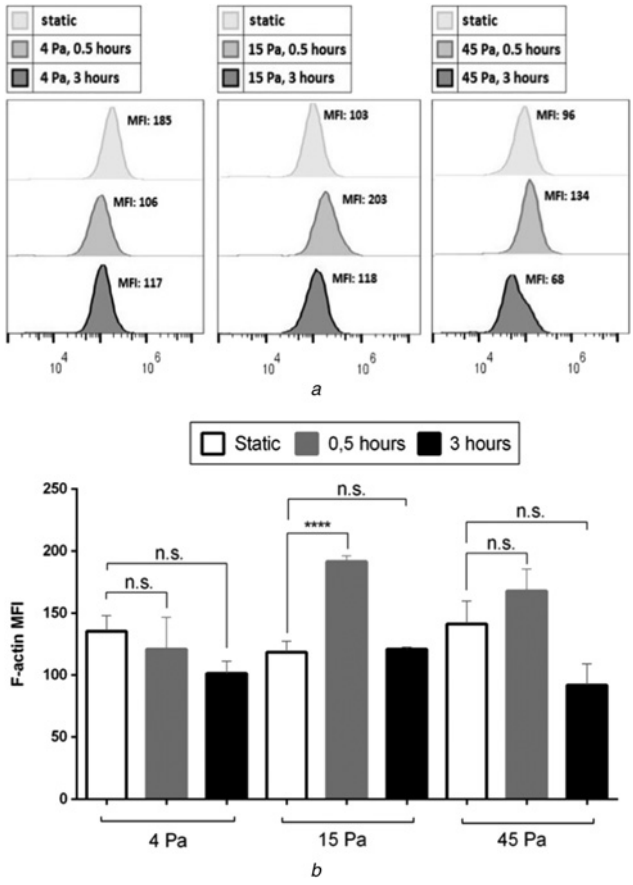

Fig. 4 *Comparison of F-actin content of THP-1 cells under static and shear stress conditions by flow cytometry. THP-1 cells were kept under static or various shear stress conditions (4, 15 and 45 Pa) for 0.5 and 3 h (n = 2 per treatment group; two independent experiments). Cells were fixed, permeabilised and stained with phalloidin – FITC. Samples (10,000 cells) were analysed by flow cytometry*

a Representative histograms of log FL-1 against events for THP-1 cells under various experimental conditions were shown. MFI values for each group were indicated

b MFI results represent the mean ± SEM of two experiments analysed by ANOVA

content of F-actin was detectable after 0.5 h of 4 or 45 Pa shear stress treatments, but was greatest 0.5 h after the treatment of 15 Pa (Figs. 3*b* and *c*) compared with static group. It is noteworthy that, with 45 Pa shear stress, which was the highest value that was applied on to the cells, caused a significant decrease of the polymerisation of actin after 3 h treatment (Figs. 3*a* and *c*).

Next, we quantitated the content of F-actin in static and shear stress applied (4, 15 and 45 Pa) groups by flow cytometry (Figs. 4*a* and *b*). No increase was observed in polymerisation of actin in THP-1 cells treated with 4 Pa shear stress at 0.5 or 3 h, contrary to microscopy data. However, we could confirm a significant increase in content of F-actin with 15 Pa shear stress at 0.5 h time point (Fig. 4*b*). Moreover, a slight increase at 0.5 h and a slight decrease at 3 h in the F-actin content on 45 Pa shear stress-treated THP-1 cells were observed compared with static group. These differences were not significant.

Overall, our data indicates that a medium level shear stress (15 Pa) increase the F-actin content that is more pronounced after 0.5 h treatment.

4. Conclusion: We show that medium and high shear stress causes profound increase of F-actin content in human monocytic cells. Hsiai *et al.* reported that shear stress provides the dynamics and molecular constituents necessary to mediate leukocyte-EC adhesion by regulation of adhesive interactions. Adhesion molecules such as selectin and integrin mediate binding kinetics between 4 and 7 dyn/cm^2 [40]. Additionally, structural changes occur by accompanying adhesive changes in mechanics by re-organisation of actin in the cells [41].

In this Letter, we designed a circulatory system to mimic vessel structure to investigate shear stress on monocyte cells with uninterrupted cycle timing under certain fluid flow. In this system, cells are passed through cavitated microchannels in a given fluid flow by a pump to create shear stress on the circulating monocyte cells. This pump provides uninterrupted cycle by discharging and refilling the liquid into syringe in seconds. Fabricated microchannels were designed in terms of creating pressure points and providing cells to pass through increasing and decreasing shear stress areas. The shear stress amplitude (difference between wide and narrow areas) was calculated by CFD. In experimental step, F-actin content was investigated after applying shear stress under circular condition as mentioned in experimental setup section and represented in Fig. 2. 4, 15 and 45 Pa shear stresses were applied on THP-1 cells during 0.5 and 3 h, by ExiGo Pump. Our study indicates an increase in F-actin content of THP-1 cells, which were under 15 and 45 Pa shear stress at 0.5 h. Moreover, at high shear stress conditions, 45 Pa, and at 3 h time points, the polymerisation of actin decreased significantly. This decrease may be due to the effect of high shear stress for a relatively long time period. It would be important to investigate the effect of different shear stress forces at different time points between 0.5 and 3 h to have a more detailed understanding of forces on THP-1 monocytic cells. Overall, our finding is the first one, to our knowledge, to show that medium or high level of shear stress leads to a change in F-actin content of THP-1 cells that may reflect the potential behaviour of monocytes in a pathological disease condition, such as atherosclerosis.

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6 References

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