Acta of Bioengineering and Biomechanics
Vol. 12, No. 4, 2010

Original paper

Steady and disturbed flow effects on human umbilical vein endothelial cells (HUVECs) in vascular system: an experimental study

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Coronary artery disease is still one of the most important reasons of the death in the world. The endothelium is the membrane of special cells which lines the interior surface of blood vessels forming an interface between circulating blood in the lumen and the rest of the vessel wall. Endothelial cells (ECs) line the entire circulatory system, from the heart to the smallest capillary. ECs dysfunction has been linked with atherosclerosis through their response to fluid forces. ECs change their morphology when exposed to mechanical stresses. The morphological responses include reorientation, elongation, and rearrangement of adhering molecules. Atherosclerotic lesions are formed in specific arterial regions, where low and oscillatory endothelial shear stress (ESS) occur. In this study, the effects of steady and disturbed flow over human umbilical vein endothelial cells (HUVECs) at different flow rates and periods were determined. Steady flow experiments were performed at flow rate of 1000 cm^3/min for twenty four hours. Disturbed flow experiments simulating the flow in branching regions of arterial systems were carried out at flow rates of 250 cm^3/min for five hours. The results obtained testified to the morphological changes easily observed. The directional alignment of the cells was determined in the steady flow experiments. Under disturbed flow conditions we observed not only the cell movement at the stagnation point but also the polygonal cell shape downstream the flow field.

Key words: *endothelial cells, atherosclerosis, hemodynamic forces, steady and disturbed flow*

1. Introduction

Coronary artery disease is the most important cause of the death in the world. Of 290 cases of the death of 20-year old or older adults, coronaries constitute 42% which far exceeds the deaths due to cancer (20%), cerebrovascular accident (11%) and other suspected causes [1]. Coronary artery disease is caused by atherosclerosis, characterized by the accumulation of lipids and fibrous elements in the arteries, making large arteries too narrow and too hard. Endothelial cells (ECs) that line the inner surface of blood vessels are continuously exposed to fluid frictional force (shear stress) induced by blood flow in the human body [2].

Vascular endothelial cells line the inner surface of blood vessels and serve as a selective barrier between the blood and other tissues and organs. It is a metabolically active monolayer constantly exposed to both biochemical and biomechanical stimuli. It is well established now that the transduction of these stimuli alone or in combination with the endothelium determines the physiology or pathology of the cardiovascular system [3].

Cell–cell junctions are the principal pathways for the transport of water and hydrophilic solutes whose particles are smaller than these of albumin. Albumin itself likely passes through the endothelium in a variety of pathways, including intercellular junctions, leaky junctions, and vesicles. Low-density lipoprotein (LDL) and high-molecular-weight materials have a limited

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Received: June 26th, 2010
Accepted for publication: December 18th, 2010

access to the normal intercellular junction and have to make use of leaky junctions or vesicles to cross the endothelium. Each of these transport pathways can be influenced by mechanical forces of fluid, particularly shear stress, acting on endothelial cells [4].

Shear stress, a tangential component of hemodynamic forces, activates many signal transduction pathways in vascular endothelial cells [5]. The studies of the effect of mechanical stress on ECs started in the 1980s. The cone and plate flow system and parallel plate flow chamber are widely used experimental apparata to apply flow shear stress (FSS) to cultured cells. In 1981, an in vitro system called *cone and plate flow system* was developed by DEWEY et al. [6] to study shear stress effects on ECs. They cultured monolayers of bovine aortic endothelial cells (BAECs) being exposed to a laminar shear stress of 5–10 dynes/cm^2 and found that confluent monolayers underwent a time-dependent change in cell shape from polygonal to ellipsoidal and became oriented with flow. DEWEY et al. observed that BAECs cultured under static conditions exhibited random stress-dependent fiber orientation. After being exposed to 8 dynes/cm^2 for 72 hr, these cells became aligned with the flow direction. Stress-subjected fibers, as visualized with both actin and myosin antibodies, appeared to be much prominent and showed orientation with the direction of the flow [7].

Similarly, BAECs grown on Thermanox plastic coverslips were exposed to shear stress levels of 10, 30, and 85 dynes/cm^2 for 24 hr using a parallel plate flow chamber. The results show that ECs whose orientation is the same as that the flow direction under the influence of shear stress are more elongated when exposed to higher shear stress [8].

Laminar shear stress is atheroprotective of Ecs, whereas disturbed or oscillatory shear stress near arterial bifurcations, branch ostia and curvatures are associated with atheroma [9]. Localization of atherosclerotic lesions to these areas is caused partly by mechanical regulation of vascular endothelial cell function by hemodynamic forces [10].

DEPAOLA et al. [11] produced a disturbed-flow region that included both flow separation and reattachment. In the near reattachment regions, shear stress was small but its gradient was large. The cells were subjected to the laminar shear stress of the magnitude of 25 dynes/cm^2 for 48 hours. Cells migrated away from this region, predominantly in the downstream direction. Depaola et al. speculate that large shear stress gradients can induce morphological and functional changes in the endothelium in the regions of disturbed flow in vivo and thus may contribute to the formation of atherosclerotic lesions. CHIU et al. [12] detail the effects of disturbed flow conditions on cultured human umbilical vein endothelial cells (HUVECs) by using a vertical-step flow channel (VSF). They demonstrate that HUVECs in the reattachment areas with low shear stresses are generally rounded in shape. In contrast, the cells under higher shear stresses are significantly elongated and aligned with the flow direction, even those in the area with reversed flow. Cheng-Nan Chen et al. (2009) constructed a parallel plate EC/SMC coculture flow system capable of producing disturbed flow and used this system to elucidate the roles of SMCs and disturbed flow in white blood cell adhesion and transmigration. When they cocultured ECs with SMCs, they found that the adhesion of neutrophils, PBLs, and monocytes to ECs under VSF prominently increased, especially in the flow reattachment area. Their findings provided new insights into the mechanisms of the interactions between WBCs and the vessel wall under the complex flow environments found in the regions of the prevalence of atherosclerotic lesions.

2. Methods and materials

2.1. Definition of endothelial shear stress

Endothelial shear stress (ESS) is the tangential stress derived from the friction of the flowing blood on the endothelial surface of the arterial wall and is expressed in units of force/unit area (N/m^2 or Pascal (Pa) or dyne/cm^2; 1 N/m^2 = 1 Pa = 10 dynes/cm^2). ESS is proportional to the product of the blood viscosity (μ) and the spatial gradient of blood velocity at the wall (ESS = $\mu\, dv/dy$), [13], figure 1.

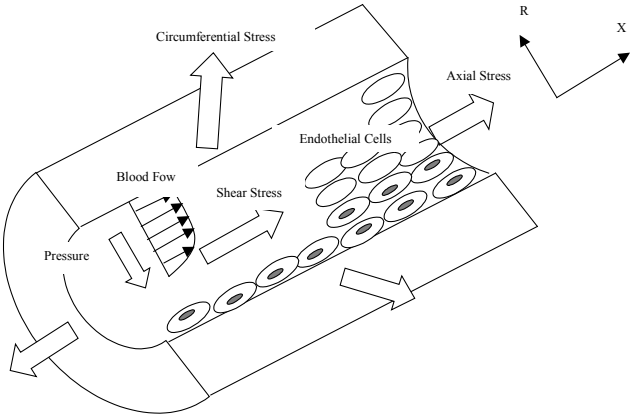


Fig. 1. Schematic of arterial wall showing endothelial cells and blood flow

2.2. Cell culture

HUVEC cell line was obtained from GATA (Gulhane Military Medicine Academy, Hematology Department, Ankara, Turkey) and cultured in Dulbecco's Modified Eagles Medium (DMEM; BIOCHROM) supplemented with 10% Fetal Bovine Serum (FBS, BIOCHROM) and 1% penicillin – streptomycin (INVITROGEN). Cells were inoculated into 0.2% gelatin-coated 75 cm^2 culture flasks and incubated in 5% CO_2 incubator at 37 °C for continious cell culture. Then, cells were subcultured twice a week by detaching the monolayer with 0.25% trypsin/EDTA (INVITROGEN) solution. Harvested cells by trypsinization were inoculated on glass coverslides for each steady and disturbed flow experiments.

2.3. In vitro flow chamber

A parallel plate flow chamber made from Plexiglass material was originally designed at our Cell Mechanics Laboratory and used to expose cultured HUVEC's monolayers to fluid shear stresses (figure 2).

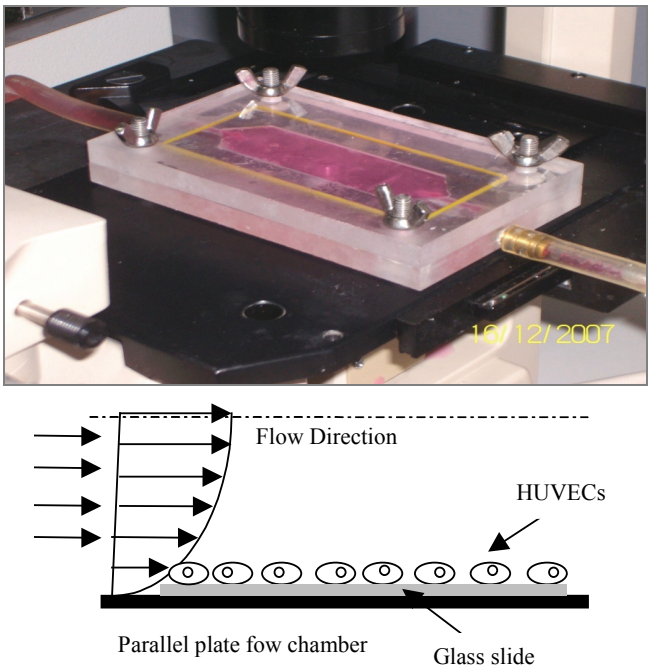


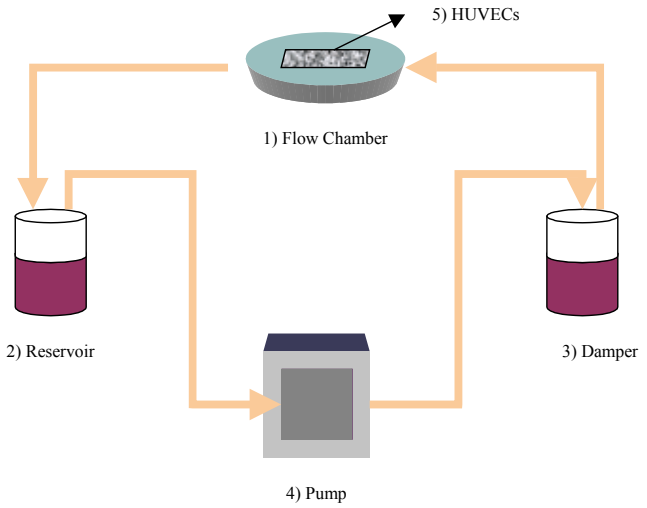
Fig. 2. In vitro flowchart used in the experiment

The top and bottom plates' long edges width are 14 cm and short edges are 8.5 cm. The thickness of a plate is 1 cm. Coverslips are placed in this channel, 7.5 cm in length and 2.5 cm in width. The channel is insulated by an O-ring to prevent the DMEM flow leakage to the surroundings.

2.4. In vitro flow system setup

Flow system is composed of a flow chamber, two DMEM bottles supplemented with 10% FBS and 1% penicillin, streptomycin (one of them is used as reservoir, the other one is used as dampener), a peristaltic pump (Masterflex L/S; Cole Parmer Instrument Company), hose (Tygon-15cm), water bath, and inverted microscope (figure 3).

(A)



(B)

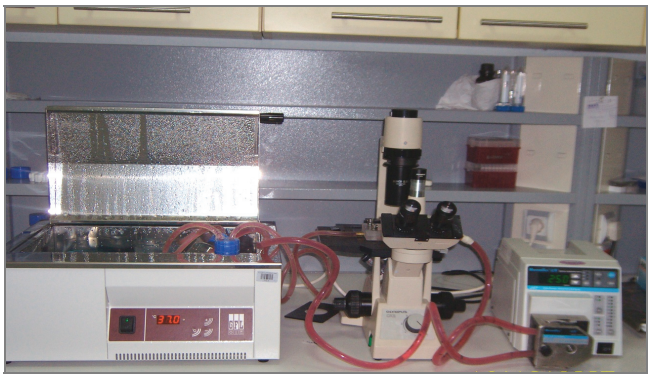


Fig. 3. Experimental setup

The pump generates the flow through the damper to the parallel plate flow chamber and back into reservoir. We sealed the lids of the damper and reservoir to prevent air flow into the bottles. The purpose of using two DMEM bottles is to prevent bubble formation. During the experiments, the system was kept at 37 °C in the water bath. A 25–75 mm coverslide was inoculared with HUVECs in Quadriperm plate (GREINER) which were cultured until reaching a confluent monolayer (2–3 days) and then gently transferred to flow chamber for experiment (figure 3). Then images under steady and disturbed flow conditions were

taken with a fluorescence microscope camera (Carl Zeiss AXIOVERT 40CFL).

3. Results

3.1. Steady flow experiments

Steady flow experiments were carried out at various flow rates in order to examine the flow rates inside different cardiovascular regions such as coronary arteries, carotid arteries, etc. Three locations over the coverslips were considered (figure 4), i.e. upper, middle and down regions. To investigate the effect of laminar shear stress on EC morphology, HUVECs were exposed to 1000 cm^3/min flow rate for 24 hours.

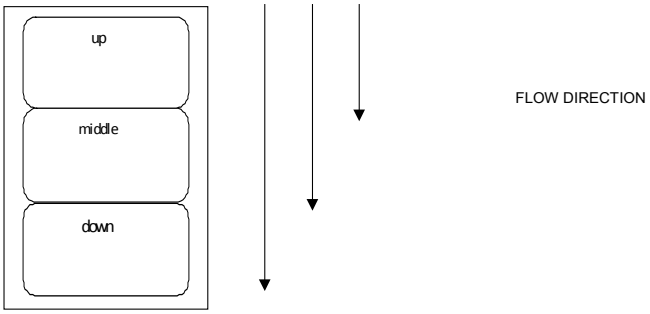


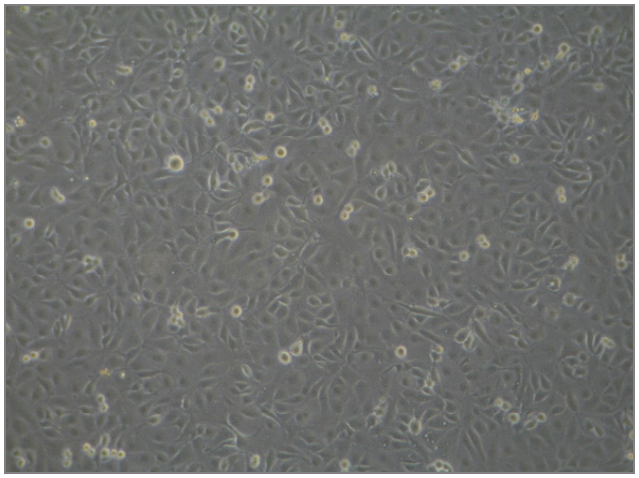
Fig. 4. Image capture locations:
upside, middle side and downside of glass slide

Shear stress was calculated by $\tau = 6\,\mu Q/(wh^2)$ inside flat plate flow chamber, where μ is dynamic viscosity (poise), Q is flow rate (cm^3/min), w is the width of the channel (mm) and h is the thickness (mm) of the flow channel. A characteristic shear stress calculation of the flow between parallel plates with the values of Q = 1000 cm^3/min, μ = 0.0084 ±0.08 poise at 37 °C, w = 2.5 cm, h = 0.2 mm will give the shear stress value of 25 dynes/cm^2 .

3.2. Steady flow experiment (Q =1000 cm^3/min, 24 hours)

Flow experiments were conducted inside the parallel flow chamber over the coverslips at the flow rate of 1000 cm^3/min for twenty-four-hour period. The morphology of the cells over the coverslips before the experiment is given in figure 5A. It is shown that the cells are polygonal in shape and form a confluent monolayer before the flow. HUVECs inoculated on coverslips are aligned in the direction of the flow by exposing to high shear stress flow. Cell alignment through flow direction was observed in the middle part (figure 5B) of coverslips.

(A)



(B)

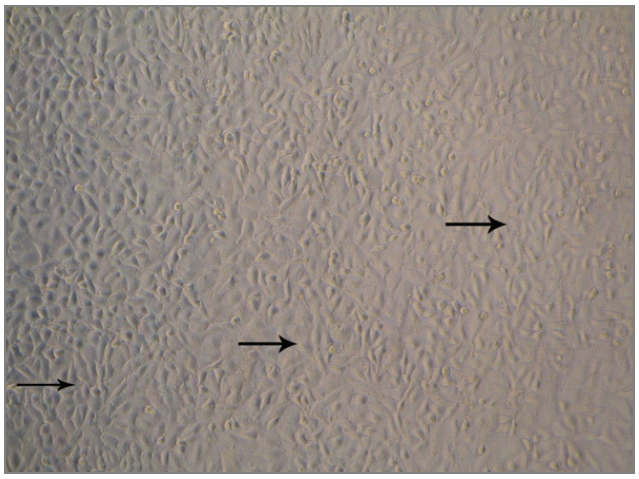


Fig. 5. Morphology of HUVECs before 24-hour steady flow experiment, upper region, 10×. Cells have a polygonal shape and form confluent monolayer (A). Morphology of HUVECs after 24-hour steady flow experiment, upper region, 10×. Arrows indicate the cell elongation along the flow direction (B). In these regions, cell shape changed from polygonal to elipsodial

3.3. Disturbed flow experiments (step flow experiments)

Atherosclerotic plaques can be found at curvatures and branches of the arterial system, where the flow is often disturbed (figure 6). Disturbed laminar flow is characteristic of the areas with reversed flow (i.e., flow separation, recirculation, and reattachment or stagnation point). The setup established to simulate the disturbed flow is shown in figure 6. The channel is 2 mm in height H and has a width of 25 mm. Two

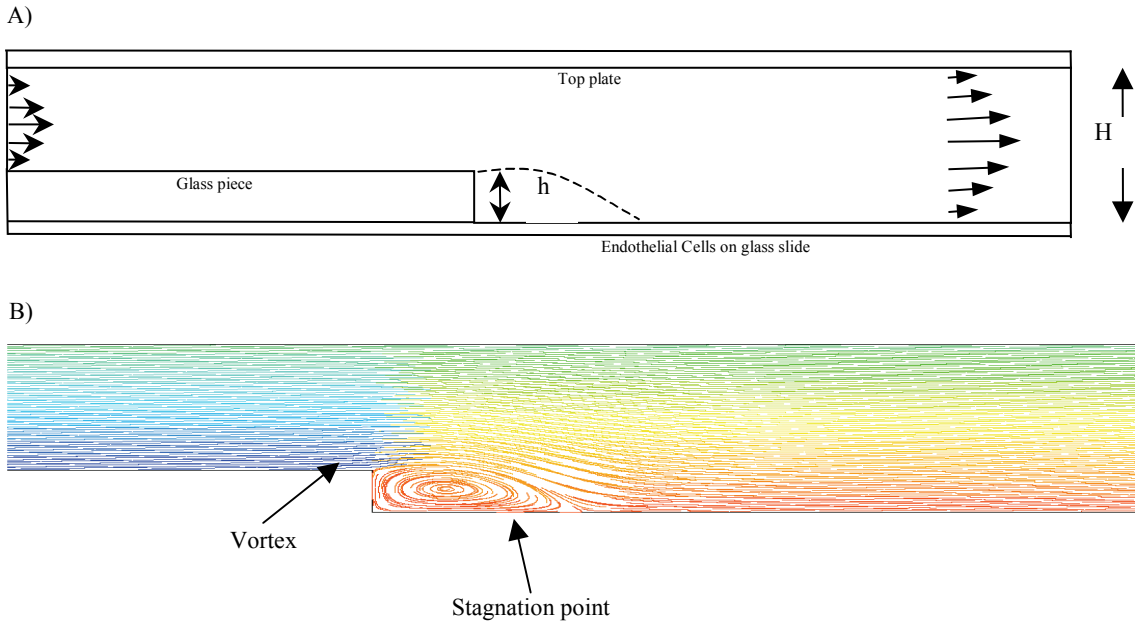


Fig. 6. Schematic presentation of step flow apparatus (A), CFD simulation model of apparatus (B)

coverslips were sticked to each other in the channel to create the step flow setup. The top coverslip thickness was 1 mm and the bottom coverslip thickness was 0.1 mm inside the channel. The difference in the height between top and bottom coverslipes was h = 0.9 mm. HUVECs were inoculated on the top of the bottom coverslip. Then fluid was allowed to flow over the cells. The computational fluid dynamics calculation (CFD) is shown in figure 6A, the flow through the channel for step flow experiment. This figure depicts the stagnation and also separation points for the experiment. Depending on the flow rate, the location of the stagnation point changes (figure 6B).

3.4. Disturbed flow experiment (Q = 250 cm^3/min, 5 hours)

Step flow experiment was done inside a parallel plate flow chamber, and the flow along the step flow model gave interesting results. Flow rate determined the reattachment site on the coverslips. Before the experiment (figure 7A) the cells had a confluent monolayer. Step is clearly seen over this region (figure 7A and 7B). After 5 hrs of the flow experiment, the loss of cells in this region was observed at the stagnation point shown in figure 7B, as cells were detached from the monolayer. Morphology of the cells in the middle region for step flow shown in figure 8A represents the results before the flow. The cells in this region (figure 8B) became rounded after the experiment.

4. Discussion

The studies conducted in vivo on animals and humans for many years suggest that hemodynamic forces influence arterial wall physiology and pathology in a number of ways [15]–[18]. It has been proposed that either very high [14] or very low shear stress [15] can contribute to vessel wall pathology. Endothelial permeability has been measured high near vessel branches and bifurcations in cardiovascular system [17], in the regions exposed to both high and low shear stresses [18]. Disruption of endothelial cell alignment has been observed in the areas where flow separation is expected to occur [19], [20], and such areas appear to be more susceptible to endothelial cell damage, intimal cell proliferation, and the development of atherosclerotic lesions [21]. The regions of the highest shear stresses are located near flow dividers which tend to prevent endothelial cell damage and lipid deposition. These in vivo observations suggest, therefore, that both the average time of wall shear stresses and temporal variations in their magnitude and direction may modify endothelial cell function.

This study presents the evidence that the application of shear stress to the surface of confluent endothelium cells in vitro can directly influence a basic endothelial cell function, a fluid-phase endocytosis. Moreover, the cells can accommodate quite rapidly to the new conditions and return to metabolic rates within several hours after the onset of shear. Data in

(A)

(B)

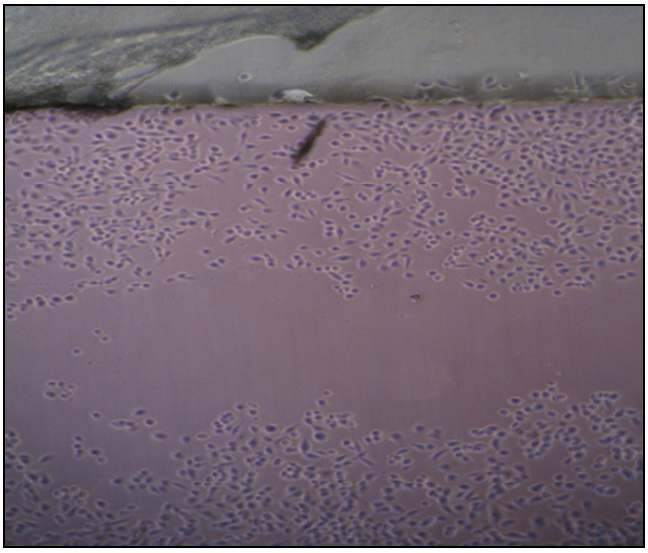
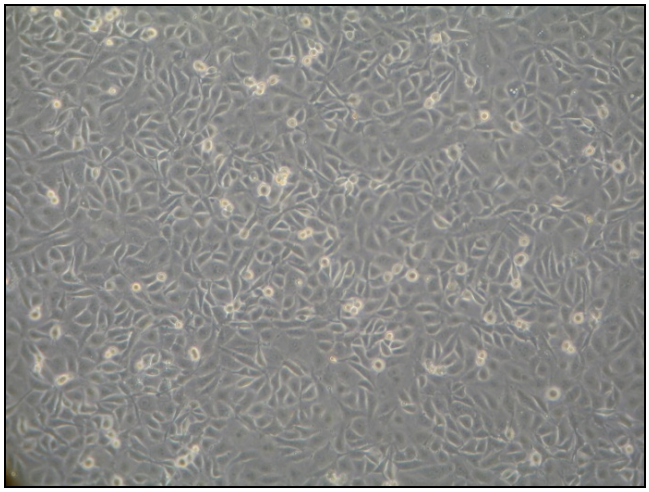


Fig. 7. Morphology of HUVECs before 5 hour step flow experiment downstream step, 10×. Cells form confluent monolayer (A). Morphology of HUVECs after step flow experiment downstream step, 5×. The region where flow falls generates a band lying below the stagnation point. In this region, cells are detached from the monolayer (B)

(A)



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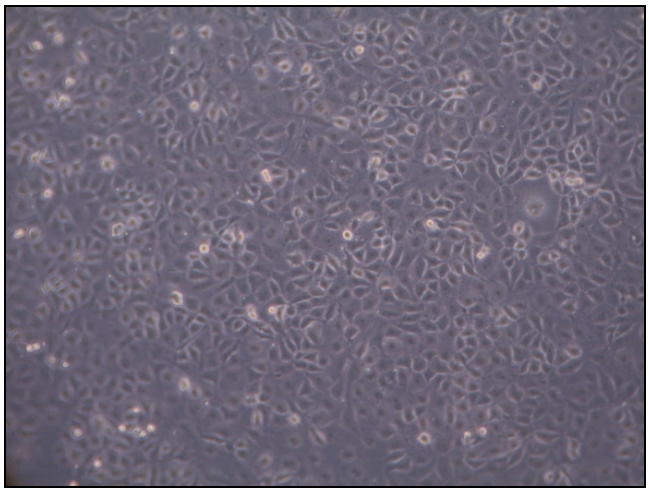

Fig. 8. Morphology of HUVECs before 5-hour disturbed flow experiment, middle region, 10× (A). Morphology of HUVECs after 5-hour disturbed flow experiment, middle region, 10×. In this region, cell become more polygonal in shape (B)

the present study refer to well-defined laminar flow conditions. In addition to laminar flow, disturbed flow occurs in regions of major arterial vessels and such forces may affect endothelial morphology and function in quite different ways than laminar flow, see figure 7B.

In summary, we have demonstrated that a basic endothelial cell function is influenced by fluid shear stresses under laminar and disturbed flow conditions, that the amplitude of the shear stress affects this response and that under certain circumstances the endothelium shows adaptation to new conditions of shear stress.

5. Conclusion

In this study, we investigated the shear stress effects on endothelial cells. We originally designed a parallel plate flow chamber in cell mechanics laboratory. Flow experiments were done using this flow chamber. HUVECs were exposed to a steady laminar flow at the rate of 1000 cm^3/min for 24 hrs. After this steady flow experiment (1000 cm^3/min), cells were elongated and aligned in the flow direction. We examined disturbed flow effects on HUVECs, designing a step model that is made by sticking two coverslips to each other. HUVECs were exposed to step flow at flow rate of 250 cm^3/min for 5 hrs. The cells were

dragged by the flow in the stagnation point or reattachment sites. In middle and down regions, cells became more polygonal. This shows that cells exposed to high flow rates die. Also, the cells are aligned in flow direction in the middle region of the coverslips. Cell shape changed from polygonal to ellipsoidal in middle region of the coverslips but cell elongation was not in the flow direction. The study will be continued in order to understand better the behaviour of protein adhesion (VE-cadherin) under different ranges of shear stresses. Additional computational simulation models will be studied at different flow rates.

Acknowledgement

First of all, we thank Fatih University for supporting our research project (Project numbers P50090803-(130) and P50070701-(396)). It would have not been possible to do this study and graduate two master students without their support. We also thank Professor Ali Uğur Ural from GATA (Ankara) for donating us HUVECs for our experiments. Finally, we would like to thank the technical staff from Fatih University for their help with the experimental setup.

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