FLUID SHEAR STRESS EFFECT ON GLYCOCALYX: MECHANOTRANSDUCTION

by

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A thesis submitted to

the Graduate Institute of Sciences and Engineering

of

Fatih University

in partial fulfillment of the requirements for the degree of

Master of Science

in

Biology

July 2008
Istanbul, Turkey

APPROVAL PAGE

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M. S. Thesis - Biology
July 2008

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ABSTRACT

Cardiovascular disease is the major cause of mortality worldwide. Hemodynamic forces such as fluid shear stress influences many physiological and pathophysiological processes of the cardiovascular system.

Also, Shear stress, is the stress component acting parallel to a given surface, like a frictional force, activates many signal transduction pathways in vascular Endothelial cell (EC) Shear stress produced by fluid mechanical forces can directly influence EC structure and function

The endothelium, a single layer of cells that lines all blood vessels, and it is the principal recipient of hemodynamic shear stress. In arteries, shear stress is strongly implicated in the localization of atherosclerotic lesions. Atherosclerotic plaques tend to be localized at sites of branching and artery curvature where the local flow is often disturbed and irregular

Mechanotransduction is a mechanism that transduce mechanical forces into cellular responses. Shear stress mechanosensors on EC include membrane receptor kinases, integrins, G-proteins, ion channels, intracellular junction proteins, membrane

lipids, cytoskeleton and also glycocalyx. Shear stress have profound effects on endothelial cell morphology, cytoskeleton organization, membrane mechanical properties, intracellular signaling, endocytosis, cell cycle entry, activation of ion channels, protein synthesis and mobilization of intracellular calcium

In this study we originally designed a flow chamber to examine the steady flow and disturbed flow that changes EC morphology at different flow rates and time periods and also degraded the endothelial glycocalyx by specific GAG enzymes called Heparinase III and chondrotinase ABC to understand whether removing glycocalyx can affect shear-induces cell responses.

After our studies we found that cell morphology changed dramatically. With steady flow experiments we observed cell aligned and elongated in the direction of flow. After disturbed flow experiments, we saw that our cells detached from the region like a long band that flow fallen down influence of step height and flow rate.

Shear stress experiments that was done with human cells had been done firstly in Turkey and got remarkable results.

Keywords: Shear stress, atherosclerosis, glycocalyx, Mechanotransduction

AKIŞ GERİLİMLERİNİN GLİKOKALİKSLER ÜZERİNE ETKİSİ: MEKANİK-İLETİM

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Temmuz 2008

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ÖZ

Kardiovasküler hastalıklar dünya da en çok ölümlere neden olan hastalıklar arasındadır. Kardiovaskülar sistemin bir çok fizyolojik ve patofizyolojik süreçleri akış gerilmeleri gibi hemodinamik kuvvetlerden etkilenir.

Akış gerilimi birçok sinyal iletim mekanizmasını aktive eden sürtünme kuvveti gibi yüzeye paralel uygulanan bir gerilim bileşenidir. Mekanik akış kuvetlerinden oluşan akış gerilimleri endotel hücrelerinin yapısı ve fonksiyonunu direk olarak etkilemektedir.

Endotelyum bütün kan damarlarını astarlayan tek bir hücre katmanıdır ve akış gerilimlerinin başlıca alıcısıdır. Arterlerde, akış gerilimi arterosklerotik lezyonların localize olmasını sağlamaktadır. Atherosklerotik plaklar sıklıkla kan akışının bozulduğu ve düzensiz olduğu arterlerin dallandığı ve eğim oluşturduğu bölgelerde localize olma eğilimindedirler.

Mechanotransduction, mekanik etkileri hücresel tepkilere dönüştürme mekanizmasıdır. Endothel hücrelerin akış gerilim mekanosenörleri membran reseptör kinaslar, integrinler, G-proteinleri, iyon kanalları, hücre içi bağlantı proteinleri,

membran lipidleri, hücre iskeleti ve glikokalikstir. Akış gerilimlerinin endotel hücre morfolojisi, hücre iskeleti organizasyonu, membranın mekanik nitelikleri, hücre içi sinyal iletimi, endositoz, hücre döngüsü, iyon kanallarının aktivasyonu, protein sentezi ve hücre içi kalsiyum salgılanması gibi hücresel fonksiyonlar üzerinde önemli bir etkisi vardır.

Bu çalışmada farklı akış hızları ve zaman periyodlarında hücre morfolojisini değiştiren sabit ve düzensiz akış deneylerini yapabilmek için laboratuvarımızda akış kiti dizayn ettik. Ayrıca glikokaliksleri endothel hücre yüzeyinden Heparinase III ve Chondrotinase ABC enzimleriyle yok ederek glikokaliklerin akış gerilmesi nedenleri hücre tepkilerinde etkili olup olmadığını anlamaya çalıştık.

Çalışmalarımızın sonunda hücrelerimizin morfolojisinde önemli değişiklikler olduğunu gördük. Sürekli akış deneyleri sonunda hücrelerimizin akış yönünde uzadıklarını, düzensiz akış deneyi sonunda ise oluşturduğumuz step in yüksekliği ve akış hızının etkisiyle akışın düştüğü bölgedeki hücrelerimizin uzun bir band şeklinde kalktığını gördük.

İnsan hücreleriyle yapılan bu tür akış gerilmesi deneyleri ülkemizde ilk defa yapılmıştır ve kayda değer sonuçlar elde edilmiştir.

Anahtar Kelimeler: Akış kayma gerilmesi, arterosklerosis, glikokaliks

Dedicated to my parents and my fiance

ACKNOWLEDGEMENT

I would like to thank my advisor Asisst. Prof. Dr. Nurullah Arslan and co-advisor Assist. Prof. Dr. Sevim Işık for their continous support and patience. Their experiences became useful for me during my experiments and writing of this thesis. I am very grateful to my labmate Berrak Tanrısever for her friendship and support.

I would also like to thank to The Graduate Institute of Sciences and Engineering of Fatih Universty for funding my thesis project P50090830-1.

Also, I want to thank my colleagues that working in Prof. Dr. Fatih Özkaragöz Cell culture laboratuvarie and my other friends in the university

Finally, I thank my family for their patience and encouragements. My special thanks to my fiancé, Semih Uykan for his love and motivation that give me.

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LIST OF SYSMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

A-II	:	Angiotensin – II
BAEC	:	Bovine Aortic Endothelial Cells
CAM	:	Cell Adhesion Molecule
CC	:	Actin cytoskeleton
CD144	:	VE-Cadherin
CS	:	Condrotin Sulfate
DMEM	:	Dulbelco's Modified Eagles Medium
ECs	:	Endothelial Cells
ET	:	Endothelin
FBS	:	Fetal Bovine Serum
GAG	:	Glycosaminoglycans
HA	:	Hyaluric Acid
HS	:	Heparan Sulfate
HUVEC	:	Human Umbilical Vein Endothelial Cells
Ig	:	Immunoglobulin
IF	:	Intermediate Filaments
MAP	:	Microtubule Associated Proteins
NO	:	Nitric Oxide
PECAM	:	Plated / Endothelial Cell Adhesion Molecule

PGI_2	:	Prostacyclin
PI3K	:	Phosphatidyinositol 3' Kinase
Re	:	Reynolds number
SH2	:	Src Homology 2
VE-Cad	:	Vascular endothelial Cadherin
ZO-1, 2	:	Zonula Occluden-1 and 2

b	:	channel width
h	:	height of the chamber
L	:	entrance length
τ	:	Shear stress
μ	:	Dynamic viscosity
Q	:	volumetric flow

CHAPTER 1

INTRODUCTION

The human cardiovascular system consists of the hearth and the blood and lymphatic vessels in the body. When blood flows, the vascular wall is exposed to hemodynamic forces of the system and disruptions in blood flow thus generate diseases of the heart and the entire blood vessel system (arteries, capillaries, and veins) within a person's body. Cardiovascular disease is the major cause of mortality worldwide and notwithstanding many efforts to reduce cardiovascular disease burden (Gouverneur, M., 2006). There are about 60 types of cardiovascular diseases. Some common types of them include;

- Atherosclerosis (hardening of the arteries)
- Coronary heart disease
- Angina
- Heart attack
- Stroke
- High blood pressure (also known as hypertension)
- Congestive heart failure.

(Schoenstadt, Arthur MD, 2006)

Hemodynamic forces in arterial tree play an important role in the formation of cardiovascular disease such as atherosclerosis.

Atherosclerosis, a disease characterized by the accumulation of lipids and fibrous elements in the arteries and cause large arteries to narrow and harden. Atherosclerotic plaques can develop in any blood vessel, but the most common locations for narrowing and hardening of the arteries include the: Heart , Brain , Legs, pelvis or arms , and Kidneys.

Arteries carry oxygen-rich blood from the heart to other parts of the body. Plaque is made up of fat, cholesterol, calcium, and other substances. As it grows, the plaque narrows the inside of the artery and may restrict blood flow.

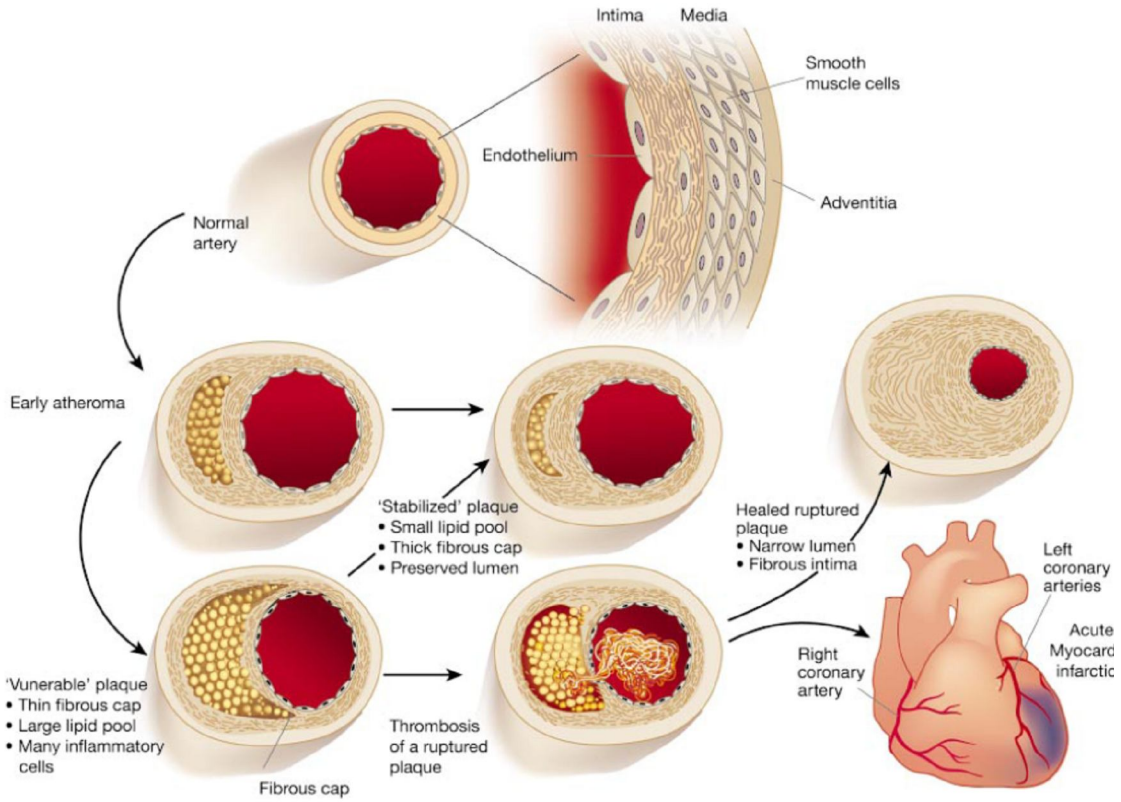


Figure 1.1 Schematic of the life history of an atheroma. (Libby, Peter 2002)

Formation of atheroma originate at sites where complex flow patterns exist in blood vessels, Figure 1.1 Blood vessel branching, bifurcation and curvature create dynamic environments of disturbed flow where flow separation and recirculation occur. Localization of atherosclerotic lesions to these areas is caused partly by mechanical regulation of vascular endothelial cell function by hemodynamic forces (V.Z.McKinney et al., 2005). These regions would have experienced complex blood flow such as elevated or reduced fluid shear stresses as shown in figure 1.2.

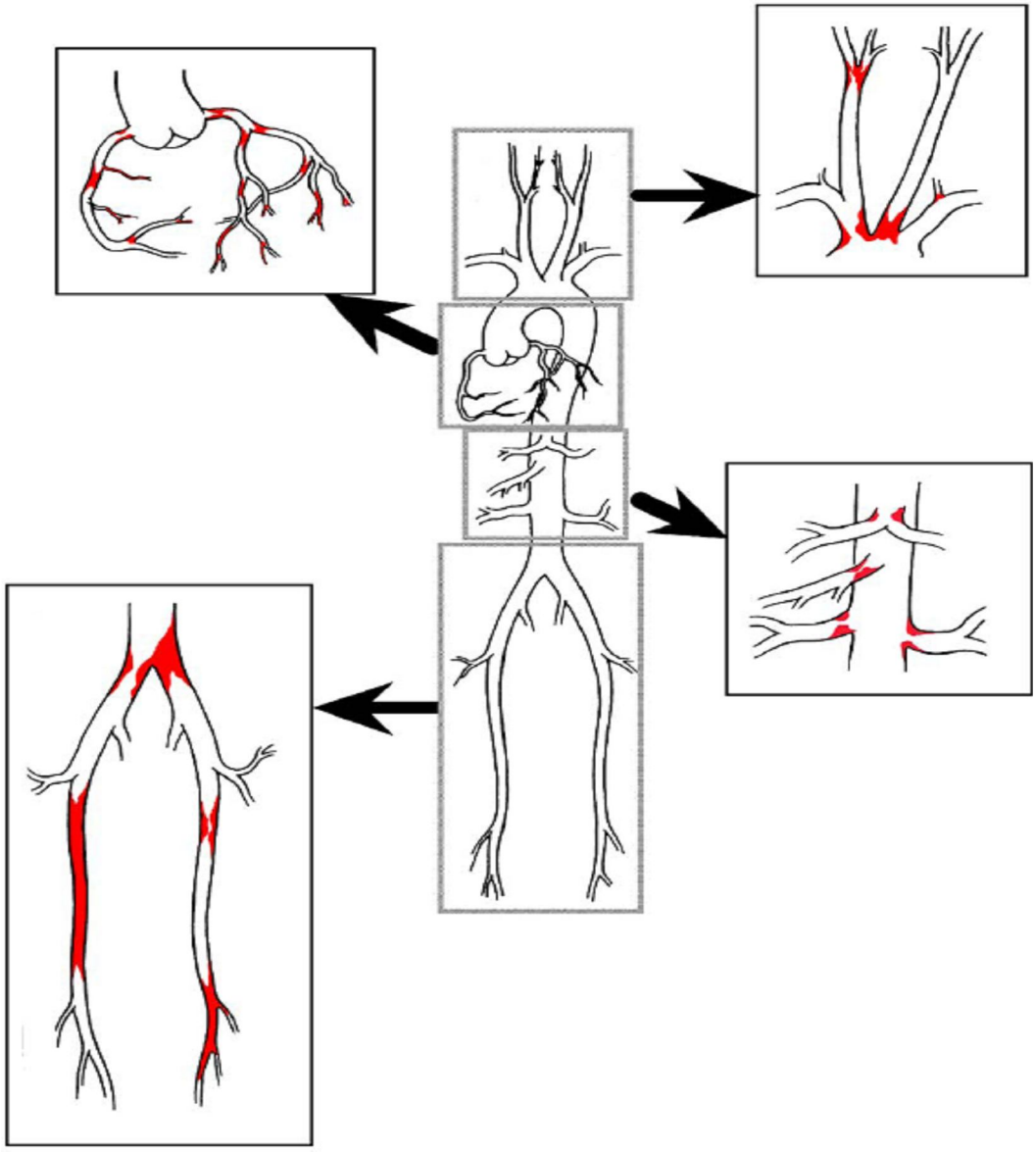


Figure 1.2 Major branches and localization of atherosclerotic legions of human arterial trees (Ohashi T, Sato M., 2003)

The frequency of clinical manifestations of atherosclerosis in Great Britain, west of Scotland in particular, is especially high. The same is true of Finland, in particular, and Scandinavia in general. Russia and many of the former states of the Soviet Union have recently experienced an exponential increase in the frequency of coronary heart disease that likely is the result of widespread economic hardship and social upheaval, a high prevalence of cigarette habituation, and a diet high in saturated fats. The frequency of coronary heart disease in the Far East is significantly lower than that documented in the West.

The rate of coronary artery disease in ethnic immigrant populations in the United States approaches that of the disease in whites, supporting the role of these putative environmental factors. Atherosclerotic cardiovascular disease is also rare on the African continent, although growing evidence indicates that this too is changing as a result of rapid westernization and urbanization of the traditionally rural and agrarian African populations. (Boudi, FB MD, 2006)

Risk factors for atherosclerosis include:

- Age (risk increases with age)
- Having close relatives with heart disease at younger ages
- High cholesterol levels, also called hypercholesterolemia
- High blood pressure, also called hypertension
- Diabetes
- Being overweight or obese
- Lack of physical activity
- Cigarette smoking.

1.1 STRUCTURE OF ENDOTHELIAL CELLS

The endothelium is a single-thin layer of cells that line interior surface of all blood vessels, forming an interface between flowing blood in the lumen and the rest of the vessel wall. The outermost layer of the vessel wall that consist of fibroelastic connective tissue is tunica adventitia and innermost layer of the endothelium is called tunica intima contains sub endothelial fibroelastic connective tissue that provides flexibility and stability to endothelial basal lamina. The middle layer, tunica media links them to a basement membrane of connective tissue comprised mainly of elastin, collagen, and smooth muscle cells as shown in Figure.1.3.

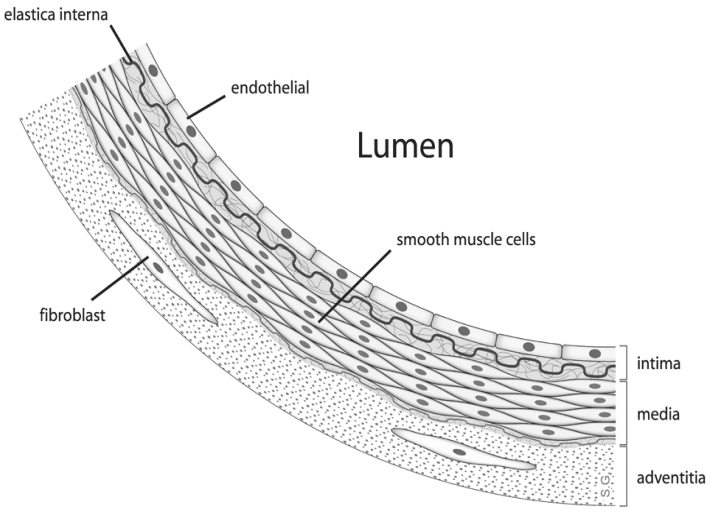


Figure 1.3 Vessel structure

Endothelial cells (ECs) are 0.2-0.5 µm thick, 10-15 µm wide and 25-50 µm long and have a centrally located oval or round nucleus. The endothelium provides a negatively charged, non-thrombotic surface that minimizes interactions with blood cells. In the circulation, ECs are constantly exposed to hemodynamic forces that modulate their functions and structure. ECs are usually flat and aligned in the direction of the flow. (Sumpio et al., 2002)

ECs perform a wide variety of important functions. These functions are;

- Synthesizing substances such as thrombosis related agents, immune response agents, vasoactivator substance, and cytokines.
- Provision of non-adherent surface for leukocytes and platelets;
- Formation of the collagen, elastic fibers and proteoglycans
- Control of exchange of nutrients and fluid between plasma and the artery wall;
- Vasocontruction and vasodilation to regulate vascular tone by releasing small vasodilatory molecules such as nitric oxide (NO) and prostacyclin (PGI_2), endothelin (ET) and angiotensin –II (A-II).
- Regulation of procoagulant and anticoagulant activities.
- Angiogenesis (new blood vessel formation)
- Protection against atherosclerosis.

1.1.1 The Cytoskeleton

The cytoskeleton is responsible for cell shape, motility of the cell and organelles within a cell. It also enables cells to perform different internal functions such as transporting intracellular vesicles and proteins. Filamentous actin (F-actin), microtubules and intermediate filaments that generate cytoskeleton, are composed of proteins that have the unique property of being able to self-assemble into a filamentous network. The cytoskeleton is a key regulator in maintaining endothelial integrity and in restoring integrity following injurious denudation, such as those that occur in the pathogenesis of atherosclerosis. **(**Joseph LT, Avrum G et.,al,2003)

1.1.1.1 Filamentous actin (F-actin)

The microfilament system are approximately 7 (nm) in diameter and important for anchoring membrane proteins, for producing cell movement and cell division. Inside cells, actin exists in two states, the monomeric protein, called G-actin (for globular actin) and the 6 nm filament, called F-actin (for filamentous actin). The factor that determines the relative proportions of F-actin and G-actin is the concentration of actin protein (Alberts, Bruce et al,. 2000). G-actin polymerizes into helix structure to form F-actin filaments shown in figure1.4.

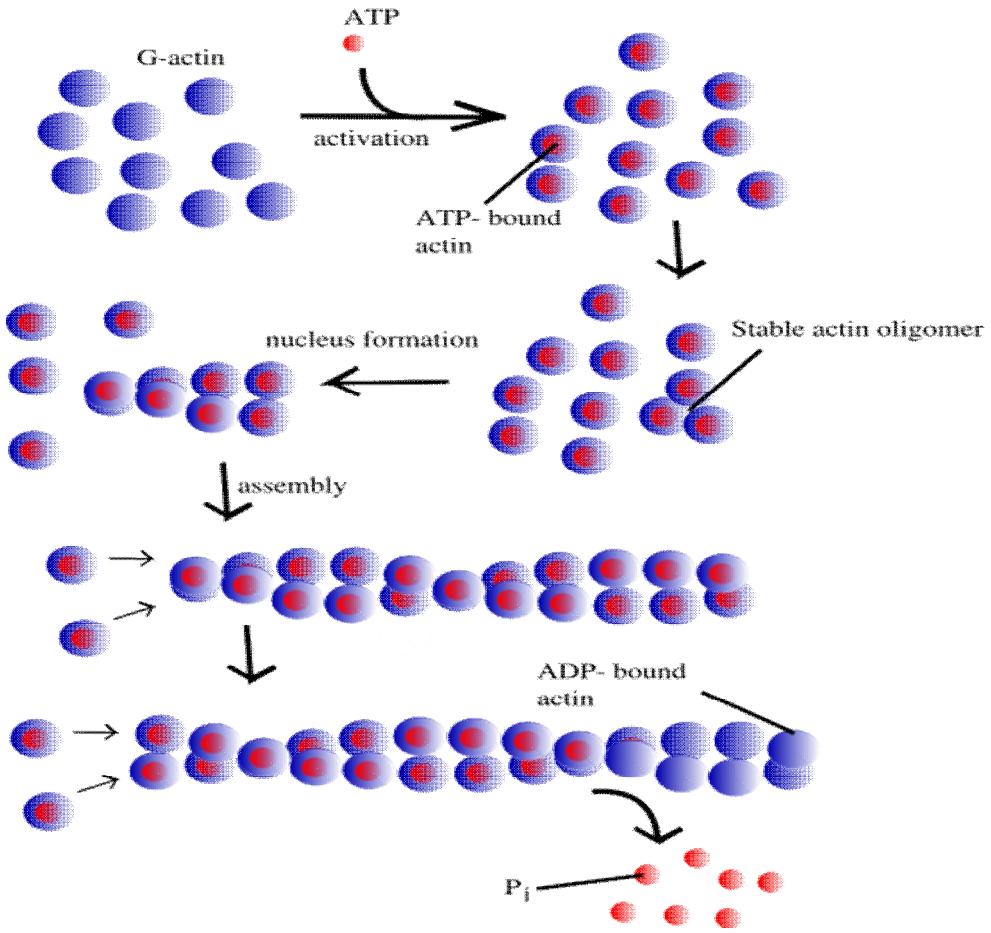


Figure 1.4 Formation of F-actin filament. (Walter F., Medical Physiology: A Cellular And Molecular Approaoch,2003)

Actin cytoskeleton organization is regulated by Rho, Rac and Cdc42 GTPases. Rho and Rac, two members of the Ras-related superfamily of small GTPases, regulate the polymerization of actin to produce stress fibers and lamellipodia, respectively. Cdc42, another member of the rho family, triggers the formation of a third type of actin-based structure found at the cell periphery, filopodia. (Nobes D., 1995). Also F-actin fortifies adherens junction.

1.1.1.2 Microtubules

A microtubule that is formed of tubulin subunits is a hollow cylinder about 24 nm in diameter. A subunit is composed of α- tubulin and β-tubulin (each approximately 50 kDA). The microtubule self-assembly in living organisms is regulated by different factors: microtubule-associated proteins (MAPs) which stabilize, destabilize and crosslink microtubules diverse kinesin-like motor proteins, which organize and link microtubules, γ-tubulin ring complex which serves as a template for nucleation sites for microtubule polymerization in centrosomes (Vladimir A et.al., 2008).

Microtubule system is very sensitive to temperature and pH changes can induce rapid disassembly. Unlike microfilaments, the microtubules do not appear to associate directly with any adhesion sites. Cell division, transport of certain organelles, morphogenesis and organization in the cell are provided by microtubules. The functions of microtubules in vesicle transport and chromosome segregation are dependent on molecular motors (kinesin and dynein) that bind to and move along microtubule tracks.

1.1.1.3 Intermediate Filaments

Intermediate filaments (IFs) are the most stable, durable cytoskeletal structure. They range in diameter from 8-10 nm between that of actin microfilaments and microtubules. There are different types of intermediate filament, each constructed from one or more proteins including keratin, desmin, neurofilaments, vimentin, peripherin and lamins. The most abundant protein constructing IFs are vimentin (54kDa) and keratins (50 to 70kDa). Most types of intermediate filaments are located in the cytosol between the nucleus and the cell membrane. At the cell borders, IFs associated with hemidesmosomes and desmosomes, found in cell -matrix and cell–cell adhesion complexes (Ukropec,JA., 1999). Intermediate filaments provide mechanical strength and resistance to shear stress.

1.2.1. Cell Junctions

There are three major types of cell junctions in vertebrates include tight junctions, gap junctions, adherens junctions and desmosomes shown in figure 1.5. Cell junctions can be classified into three functional groups:

1. **Occluding junctions** seal cells together in an epithelium in a way that prevents even small molecules from leaking from one side of the sheet to the other.
2. **Anchoring junctions** mechanically attach cells to their neighbors or extracellular matrix.
3. **Communicating junctions** mediate the passage of chemical or electrical signals from one interacting cell to its partner. (Alberts, B., 2000)

1.2.1.1 Tight Junction

The tight junction or zonula occludens sites provide the membranes of two cells come very close together. Claudin and occludin proteins are the transmembrane parts of the junction, and the cytoplasm part is linked to the microfilament network (Tsukita et al., 2001)

Tight junctions control cellular permeability across epithelial cell sheets and also serve as a gatekeeper to provide diffusion of components between a cell's apical and basolateral membrane domains. The cytoplasmic proteins zonula occluden-1, 195kDa, (ZO-1) and zonula occluden-2, 160kDa, (ZO-2) complex with occludin to form tight junctions as well as possibly being involved in adherens junctions (Ukropec., JA 1999). ZO-1 binds to ZO-2 and occludin.

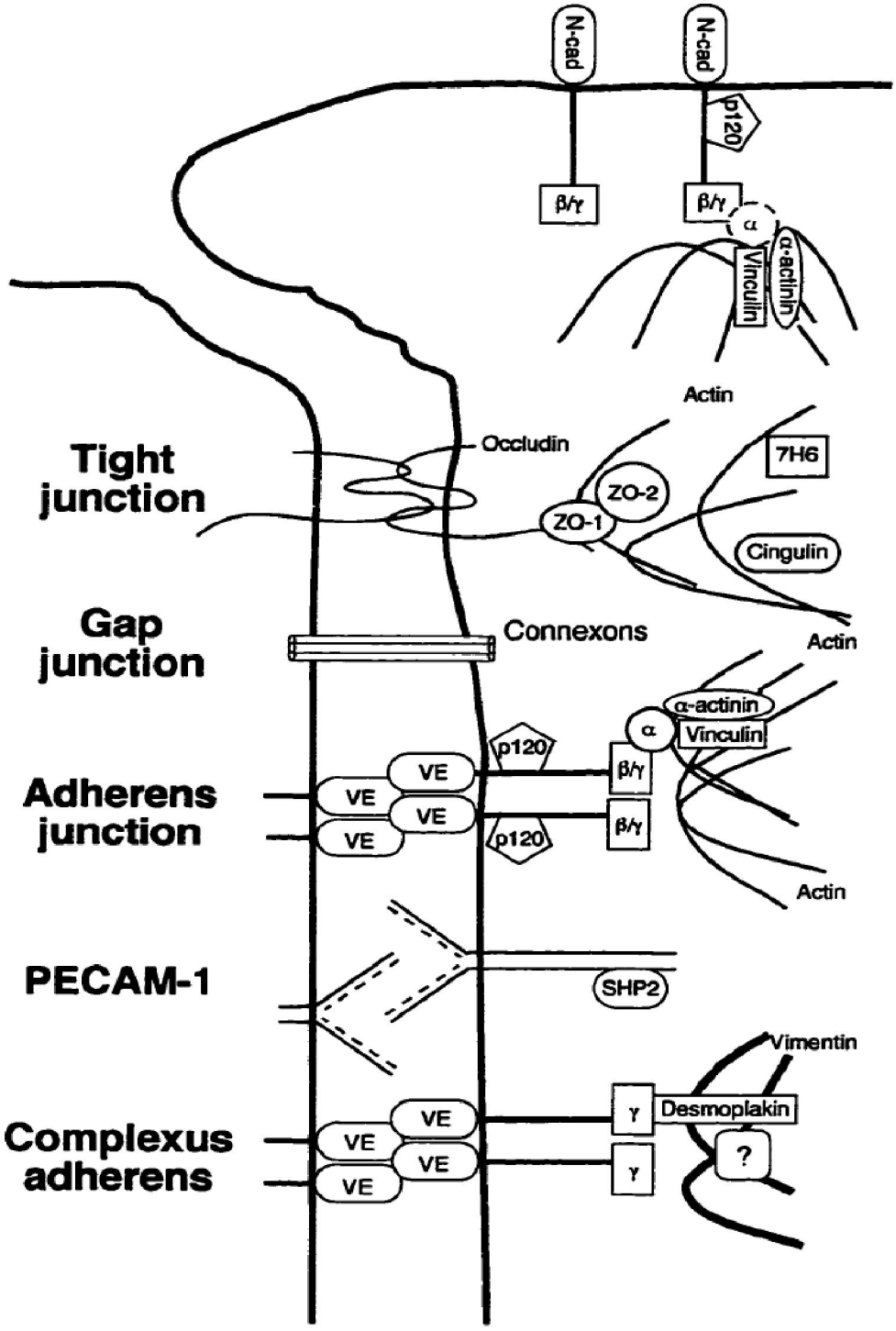


Figure.1.5 Interendothelial cell-cell junctions (Ukropec, JA., 1999)

1.2.1.2 Gap Junction

Gap junctions comprised of connexons that provide signal transduction among cells. Connexins are four-pass transmembrane proteins, six of which assemble to form a channel, a connexon. When the connexons in the plasma membranes of two cells in contact are aligned, they form a continuous aqueous channel that connects the two cell interiors. The connexons hold the interacting plasma membranes at a fixed distance apart—hence the gap (Alberts, B., 2008). Small molecules (molecular weight <1kDa) can pass through the channel of each gap junction.

1.2.2. Cell-cell Junction molecules - PECAM-1/CD31

PECAM-1(Plated/endothelial cell adhesion) is a 130kDa transmembrane glycoprotein belonging to the Immunoglobulin (Ig) superfamily of cell adhesion molecules (CAMs) primary located and expressed at cell junctions of ECs. It plays an important role EC-neuptrophil interaction and transmigration in inflammation. PECAM-1 is also known to regulate EC calcium signals and undergo selective tyrosine phosphorylation. PECAM-1 may function as an inhibitory receptor, interacting with various signaling molecules through its Src homology (SH2) containing protein tyrosine phosphates (SHP-2) binding domain. Tyrosine phosphorylation of PECAM-1 generates Src homology 2 (SH2)-binding sites that can cause association with cytosolic proteins containing SH2 domains such as the intracellular phosphatases SHP1 and SHP2 (Ukropec,JA., 1999, N. Dusserre et al., 2004). Furthermore, PECAM-1 act as a shear stress mechanosensor.

1.2.3. Adherens Junctions

The adherent junctions between endothelial cells are protein complexes that mediate calcium-dependent homophilic binding of extracellular domains of cadherins and also important for maintain a barrier between blood components and subendothelial tissues. Adherens junctions consist of cadherin complexes. The cytoplasmic domains of cadherins are linked to the actin cytoskeleton by catenin proteins (Noria, S., et al., 1999). Adherens junctions and desmosomes are responsible for mechanically coupling myoyctes in the hearth and are found closely, apposed to gap junction plaques at intercalated discs of cardiomyocytes

(Gutstein, DE et al., 2002). Adhesion mechanism is important in processes tissue morphogenesis, cell motility and cell migration.

1.2.3.1. Cadherins

Cadherins are family of transmembrane receptors that mediate calcium dependent homophilic cell-cell adhesion. This superfamily involves; 1) classical cadherins that are major component of cell-cell adhesive junctions, 2) desmosomal cadherins (desmocolins and desmogleins), 3) protocadherins, 4) some other cadherin related molecules (e.g. the fat protein of Drosophila) (Navarro, P et al., 1998). In adherens junctions, cadherins are clustered and connected through their cytoplasmic domain with a complex network of cytoskeletal proteins. Linkage of cadherins cytoplasmic domain to three cytosolic proteins, named α-catenin, β-catenin and γ-catenin (plakoglobin) (Reynolds, AB et al., 1994). The C- and N- termini of the cadherin proteins are located outside and inside the cell. The extracellular portion of the cadherin molecule comprise of five cadherin domains that begin with the N- terminus of the molecule (Ivanov, DB et al., 2001) Figure 1.6.

The N terminus of the cadherin molecule contains calcium binding regions required for formation and function of cadherins. The cytoplasmic region directly binds to three homologous proteins that belong to the 'armadillo' family (approximately 42 amino acid sequence originally described in Drosophila) include β-catenin, plakoglobin and p120 (Navarro, P et al., 1998). β and α- catenin bind to the cytoplasmic domain of cadherin and link the catenin –cadherin complex to the actin filaments. In addition, α-catenin directly binds to F-actin cytoskeleton because it is partially homologous to the actin binding protein vinculin.

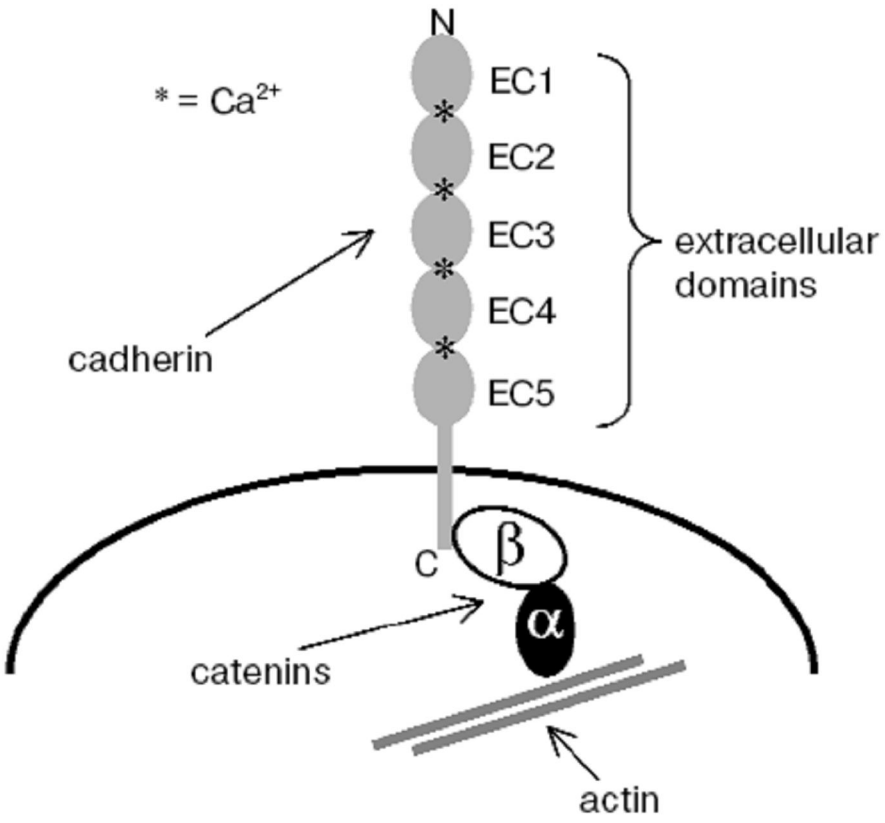


Fig.1.6 Structure of classical cadherins and their interaction with cytoplasmic proteins.

The two major cadherins of endothelial cells are neural (N)-cadherin and vascular endothelial (VE)-cadherin. Despite similar level of protein expression only VE-cadherin is located at cell–cell contacts, whereas N-cadherin is distributed over the whole cell membrane (Navarro, P et al., 1998). Cadherins have important roles in development. The best characterized cadherin is E-cadherin that is expressed in epithelial tissues and regulate cell-cell adhesion, cell migration, morphogenesis and regulation of membrane polarity. It also helps cause compaction, an important morphological change that occurs at the eight-cell stage of mouse embryo development. During compaction, the loosely attached cells, called blastomeres, become tightly packed together and joined by intercellular junctions (Alberts, B, 2008). While VE-Cadherin is expressed in endothelial cells, N- Cadherin is expressed in many different cell types like neurons, heart, skeletal muscle, lens and fibroblasts.

VE-cadherin (known as cadherin 5 and CD144) is the major adhesive protein of adherens junctions and it may transfer information intracellulary with cytoskeletal network and signal molecules. Vascular endothelial growth factor that induces tyrosine phosphorylation of VE-cadherin, generate a complex with VE-cadherin, β-catenin and PI3 kinase. Thyrosine phosphorylated VE- cadherin provides the docking sites for multiple signaling molecules including SHP2, phosphatidyinositol 3' kinase (PI3K) and Shc (Fukuhra, S et al., 2006). Extracellular domain of VE-cadherin is necessary for homotypic adhesion and clustering, the intracellular association to catenins and the actin cytoskeleton is required for the stabilization of the complex and a full control of junctional permeability (Corada, M et al., 1999).

1.2. GLYCOCALYX

A wide variety of membrane bound macromolecules constitute a negatively charged organized mesh called glycocalyx on the endothelial cell surface. Over the past decade, researchers have been investigated the important physiological functions of glycocalyx, including mechanotransduction, hemostasis, signaling, and blood cell-vessel wall interactions (Reitsma S et al., 2007).

We focused on the relationship between fluid shear stress and the endothelial glycocalyx by removing glycocalyx and then we compared the two major components of glycosaminoglycans (GAGs) in the glycocalyx, heparan sulfate GAGs and chondrotin sulfate GAGs.

1.2.1 Structural Properties of Glycocalyx

The glycocalyx that is carbonhydrate - rich layer consisting of various membranous glycoproteins, proteoglycans and glycosaminoglycans (GAGs) that include heparin sulfate (HS), Condrotin sulfate (CS) and Hyaluric acid (HA). Estimated thickness of glycocalyx ranging from 0.5micron to over 1micron into the vessel lumen. More recent studies indicate that glycocalyx thickness increases with vascular diameter, at least in the arterial system, ranging from 2 to 3 micron in small arteries to 4.5 micron in carotid arteries (Reitsma, S et al., 2007)

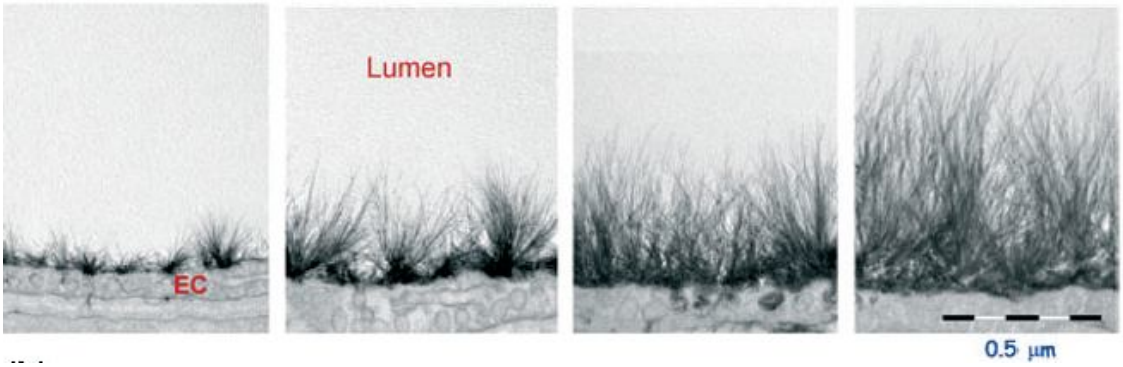


Fig.1.7 Examples of spatial heterogeneity of glycocalyx dimensions in the vascular system (Gouverneur, M et al., 2006)

Soluble plasma components that generate glycocalyx, linked to each other with soluble proteoglycans and / or glycosaminoglycans. A dynamic equilibrium placed between soluble components of glycocalyx and flowing blood. Therefore, the effective thickness and composition of the layer is dependent on the local plasma condition as well as hemodynamic environment (Yao, Yu, 2005). Syndecans are proteoglycans and proteoglycans are 'backbone' molecules of glycocalyx. They contain a core protein, one or more sulfated GAGs are covalently attached. Sydecans-1 (33kDa), -2 (22kDa), and -4 (22kDa) expressed on ECs and contain GAG attachment sites. GAGs chains composed of 100 disaccharide units is around 80 nm. Heparan sulfate (HS), Condrotin sulfate (CS), dermatan sulfate, keratan sulfate, and hyaluronan (Hyaluronic acid) (HA) are types of GAGs. The majority of GAG chains added to syndecan core proteins are of the heparan sulphate type, although syndecan-1 and syndecan-4 have been shown to be modified by CS chains as well (Carey, DJ., 1997). Heparan sulfate and chondrotin sulfate is formed in Golgi apparatus and endoplasmic reticulum (E.R). Also, cytoplasmic tails of sydecan-1 and -4 associated with F-actin cytoskeleton to transmit signals through intracellular cytoskeleton. Heparan sulfate and chondrotin sulfate are the most abundant GAGs in endothelial glycocalyx. Figure 1.8 represent all of the components of the glycocalyx.

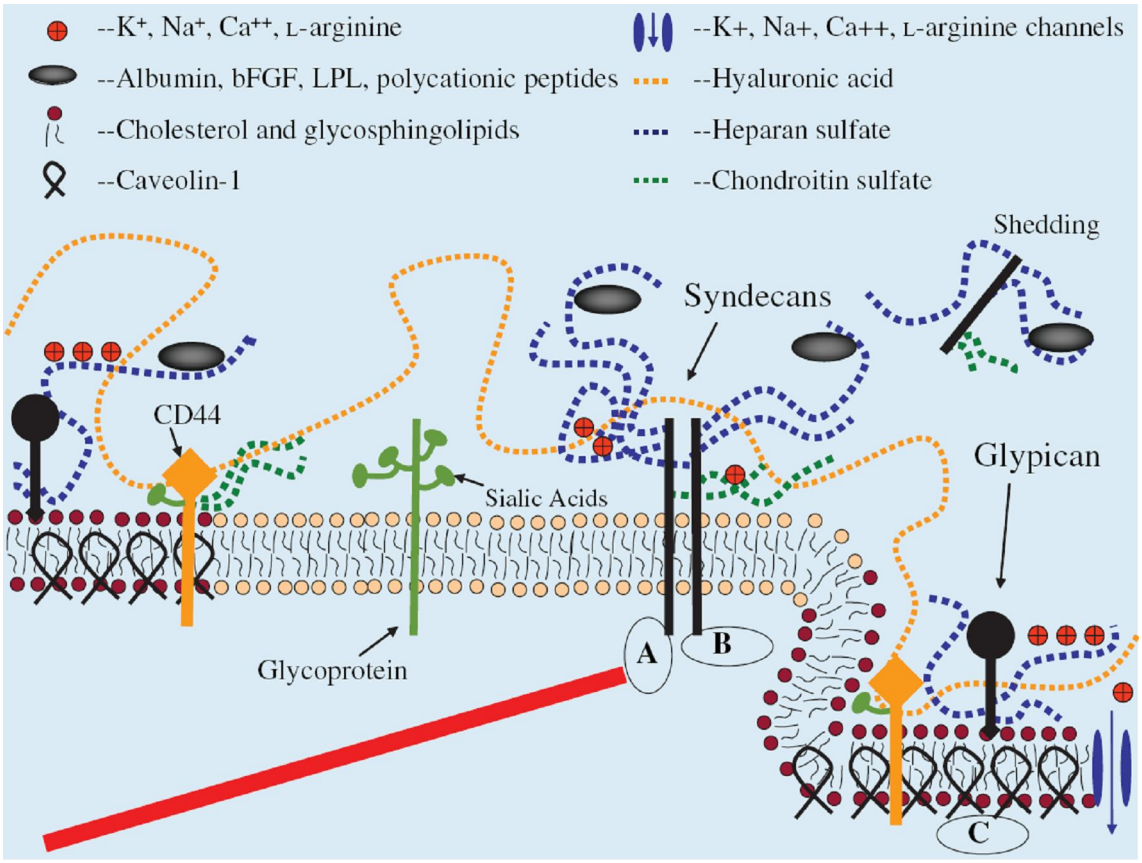


Fig.1.8 Representation of proteoglycans and glycoproteins on the surface of ECs (Tarbell JM and Pahakis MY, 2006)

Protein caveole-1 forms caveolae that is a rigid cholesterol rich membranous domain contain signal molecules. The responsiveness of these structures to shear stress was demonstrated recently by rapid movement of NO from the caveolae, where it is bind to the caveolin (Restnick N et al., 2003). Glypican is bound directly to plasma membrane and has three GAG attachment sites that localize HS. Transmembrane CD44 receptor localizes in caveolae and contains CS and HA. Glycoproteins are also backbone molecules like proteoglycans, linking the glycocalyx to the cell membrane. The level of glycoprotein expression depends on cell stimulation. The endothelial cell adhesion molecules (selectin, integrin family and immunoglobulin superfamily) are well-defined glycoproteins that play a major role in cell recruitment from the bloodstream and in cell signaling (Reistma S et al., 2007). Cytoplasmic tails of sydecans associate with α actinin, link it to the cytoskeleton (red line in the figure 1.8).

The important roles of glycocalyx are protection of the plasma membrane from chemical injury, contributing to the endothelial permeability barrier, enables sperm to bind to eggs, modulating inflammation by inhibiting leukocyte adhesion and the other function of it that interest us is mechano-shear sensor, transmit the shear stress to cytoskeleton.

1.3. LITERATURE REVIEW

1.3.1 Atherosclerosis and Hemodynamic Forces

The ability of the vascular endothelium to sense and respond to the flow was observed more than 150 years ago by the famous pathologist Virchow, who pointed to the heterogeneous morphology of the endothelium along the arterial tree, which correlated with the patterns of flow to which the cells are exposed (Resnick N et al., 2003). The endothelium, a single layer of cells that lines all blood vessels, is the focus of intense interest in biomechanics because it is the principal recipient of hemodynamic shear stress. (Helmke BP, Davies PF 2002). Fluid dynamics research over the past twenty years has contributed immensely to knowledge of atherosclerosis. Since atherosclerotic plaques tend to be localized at sites of branching and artery curvature where the local flow is often disturbed and irregular (e.g., flow separation, recirculation, complex flow patterns, and nonuniform shear stress distributions) and since these locations would be expected to harbor complex flow patterns, investigators postulated that fluid dynamics might play an initiating role in atherosclerosis (Giddens DP et al., 1993, Chiu JJ et al., 1998). Depaola et al., 1992 produced a disturbed-flow region that includes both flow separation and reattachment. Near reattachment regions, shear stress is small but its gradient is large. Cells migrate away from this region, predominantly in the downstream direction. Those that remain divide at a rate that is high compared with that of cells subjected to uniform shear. They speculate that large shear stress gradients can induce morphological and functional changes in the endothelium in regions of disturbed flow in vivo and thus may contribute to the formation of atherosclerotic lesions.

1.3.2 Shear Stress Effects on Endothelial Cells

The principal mechanical forces sensed by ECs are the shear stress of flowing blood on their apical surface, and the circumferential stress resisting blood pressure, which induce stretch in the cell body (Tarbell JM, Pahakis MY, 2006). Mechanotransduction refers to the mechanism converting physical forces into the cellular responses (Huang H et al., 2004). Shear stress, the tangential component of hemodynamic forces, activates many signal transduction pathways in vascular endothelial cells (Chen KD et al., 1999). Measurements show that shear stress ranges from 1 to 6 dyne / cm^2 in venous system and 10 to 70 dyne / cm^2 in arterial vascular system (Tai, KD, 2005).

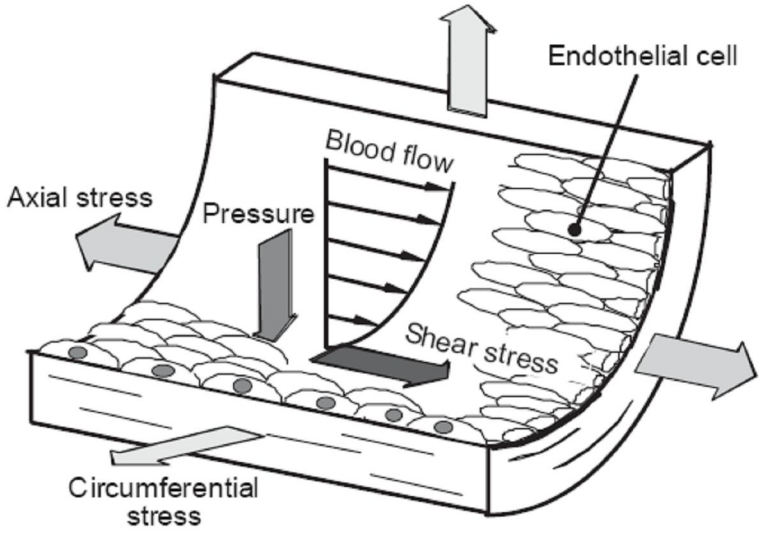


Figure 1.9 Arterial wall showing endothelial cells and blood flow (Ohashi T, Sato M., 2003)

For the vascular endothelial cell that resides at the interface of the flowing blood and the underlying vessel wall, there is mounting evidence of the importance of flow and the associated wall shear stress in the regulation of endothelial biology (Nerem RM et al., 1998).

ECs are normally exposed constantly to mechanical forces that significally influence their phenotype. From a simplistic standpoint, changes in FSS could be sensed directly by cell membrane mechanosensors/receptors, however two approaches were taken in the attempt to define a shear stress receptor in ECs – the first approach documented very early events (seconds-minutes) that occur in endothelial cells following their exposure to shear stress. The second approach links very early changes that occur in potential endothelial receptors/sensors to known signaling events, by interfering with these changes and blocking shear-stress-mediated signaling. EC shear stress mechanosensors include membrane receptor kinases, integrins, G proteins, ion channels, intracellular junction proteins, membrane lipids, and cytoskeleton (Chien et al., 2001, Resnick N et al., 2003). In recent years, with the advent of cell culture studies, much has been learned about the shear stress effects on endothelial cell morphology, cytoskeleton organization, membrane mechanical properties, intracellular signaling, endocytosis, cell cycle entry, activation of ion channels, mRNA, protein synthesis and mobilization of intracellular calcium (Yao, Yu, 2005).

Endothelial cells alter their morphology, growth rate, and metabolism in response to fluid shear stress. These include activation of ion channels and G proteins, induction of oscillations in intracellular calcium concentration, alterations in the expression of various important genes, and extensive cytoskeletal reorganization (Barakat AL, 1999). Stamatas GN and Mclntire LV studied rapid flow-induced response in the EC morphology and calcium distribution and then demonstrate that within first minutes of flow application nuclear calcium is increasing and also whole cell height and nuclear height are reduced by about 1 micron. To understand the role of the cytoskeleton in the FSS responses, they disruptered each of the cytoskeletal elements with specific enzymes such as acrylamide, cytochalasin D, and colchicine but none of these compounds had any effect on the shear- induced calcium response and colchicine completely abrogated the response (Stamatas GN and Mclntire LV, 2001)

In vitro models for the study of endothelial cell responses must consider the pattern of shear stress including steady and disturbed flow (Fisher AB et al., 2001 and Barbee KA, 2002). Dewey et al.,1981, exposed to a laminar shear stress of 5-10 dyne / cm^2 to cultured monolayers of bovine aortic endothelial cells (BAECs) using a cone –plate apparatus and found that confluent monolayers undergo a time-dependent change in cell shape from polygonal to ellipsoidal. Similarly, bovine aortic endothelial cells grown on Thermanox

plastic coverslipes 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 orient with the flow direction under the influence of shear stress and more elongated when exposed to higher shear stress (Levesque MJ and Nerem RM, 1985). C.G.Galbraith et al, determined the spatial reorganization of the cytoskeleton throughout the volume of cultured BAECs after the cells exposed to physiological level of shear stress for 0, 1, 5, 3, 6, 12, or 24 hours. They separate the cell responses three distinct phases. The first phase occurred within 3 hr and cells elongated. After 6 hour the monolayer entered the second phase that cells lost their dense peripheral bands and nuclei located in the upstream region of the cell. In the third phase after 12 hour exposure cells oriented in the direction of the flow (Galbraith CG et al., 1998). Also, ECs are capable of producing intracellular actin and myosin filaments that are oriented in the flow direction (Dewey, CF, 1984).

Davies PF et al., demostrate that the shape of cultured ECs was altered by exposure to unidirectional shear stresses of 8 dynes / cm^2 in laminar flow within 24 hr. Individual cells became ellipsoidal and the cell population assumed an axial alignment in the direction of flow. In contrast, application of a mean shear stress as low as 1.5 dynes/cm^2 for 16 hr in turbulent flow resulted in random orientation of cells in the monolayer. Prolonged exposure to turbulent flow (24 hr or longer), however, resulted in the development of gaps in the monolayer, reflecting cell retraction and cell loss (Davies PF et al., 1985).

Laminar shear stress atheroprotective for endothelial cells, where as nonlaminar, disturbed or oscillatory shear stress correlates with develop of atherosclerosis (Nerem, RM 1992). And also nonlaminar flow promotes changes to endothelial gene expression, cytoskeletal arrangement, wound repair, leukocyte adhesion as well as to vasoreactive, oxidative and inflammatory states of the artery wall (Cunningham KS and Gotlieb AL, 2005). Atheroprotective molecules such as (nitric oxide and prostacyclin), hormones (natriuretic peptides), and matrix molecules (heparans and extracellular superoxide dismutase (SOD) are produced in response to steady laminar blood flow (Yoshizumi, M et al., 2003).

1.3.3 Studies on Glycocalyx

Florian et al tested whether a heparan sulfate component of the glycocalyx is a fluid shear stress sensor on ECs. The NO production induced by steady shear stress (20 dyne / cm^2) and oscillatory shear stress (10+ / -15 dyne / cm^2) was inhibited by pretreatment with heparinase III but addition of bradykinin induced NO production was not inhibited by heparinase III. These experiments demostrate that a heparan sulfate component is a mechanosensor that mediates NO production in response to shear stress (Florian, JA et al., 2003). Pahakis et al proved that depletion of HS, HA, and SA from surface of bovine aortic endothelial cell by Heparinase III and chondrotinase ABC but not CS, blocked shear-induced NO production they surprisingly found that blocked NO production had no influence on shear induced (PGI2) production (Pahakis et al.,2007). Also Gouverneur et al. demonstrate that exposure of cultured endothelial cells for 24 hr to a shear stress of 10 dynes / cm^2 stimulates incorporation of glucosamine-containing GAGs in the glycocalyx, which is accompanied by elevated levels of glusamine-containing GAGs in the supernatant. These increases were confirmed by direct demonstration of increased hyaluronan concentration in the glycocalyx and in the supernatant, as well as by a threefold increase in the incorporation of hyaluronan-binding protein in the glycocalyx (Gouverneur, M et al., 2006).

Weinbaum S et al., proposed a structural model represents the core proteins in the proteoglycan clusters that comprise the glycocalyx and their linkage to the underlying actin cytoskeleton (CC) as shown figure 9. This composite structure is deduced from the appearance of bush-like structures that appear to emanate from foci in the cell membrane and current models of the CC. Also shown are transcellular actin stress fibers linked by α-actinin tethering the cortical shell to focal adhesion sites of integrins on the basal aspect of the cell and other tethering filaments associated with actin filament bundles in close proximity to the junctional complexes. There is a bidirectional grid with 20-nm periodicity of scattering centers aligned along the axes of the core proteins. As shown figure 1.10, there is also a 100-nm periodicity associated with the separation of each cluster and the observed hexagonal organization of the membrane bound foci (Weinbaum, S et al., 2003).

Yao, Yu et al., investigated that the role of the glycocalyx in both EC short-term and long-term responses by using heparinase III to cleave heparan sulfate GAGs on the cell surface. When they remove the glycocalyx with heparinase III, cells no longer align under flow after 24 hr and they proliferate as if there were no flow present. On the other hand,

control ECs quickly responded to the onset of laminar flow by decreasing their migration speed by 40% in the first hour and increasing the amount of vascular endothelial (VE)–Cadherin in cell – cell junctions. These responses are not observed in the cells treated with heparinase III.

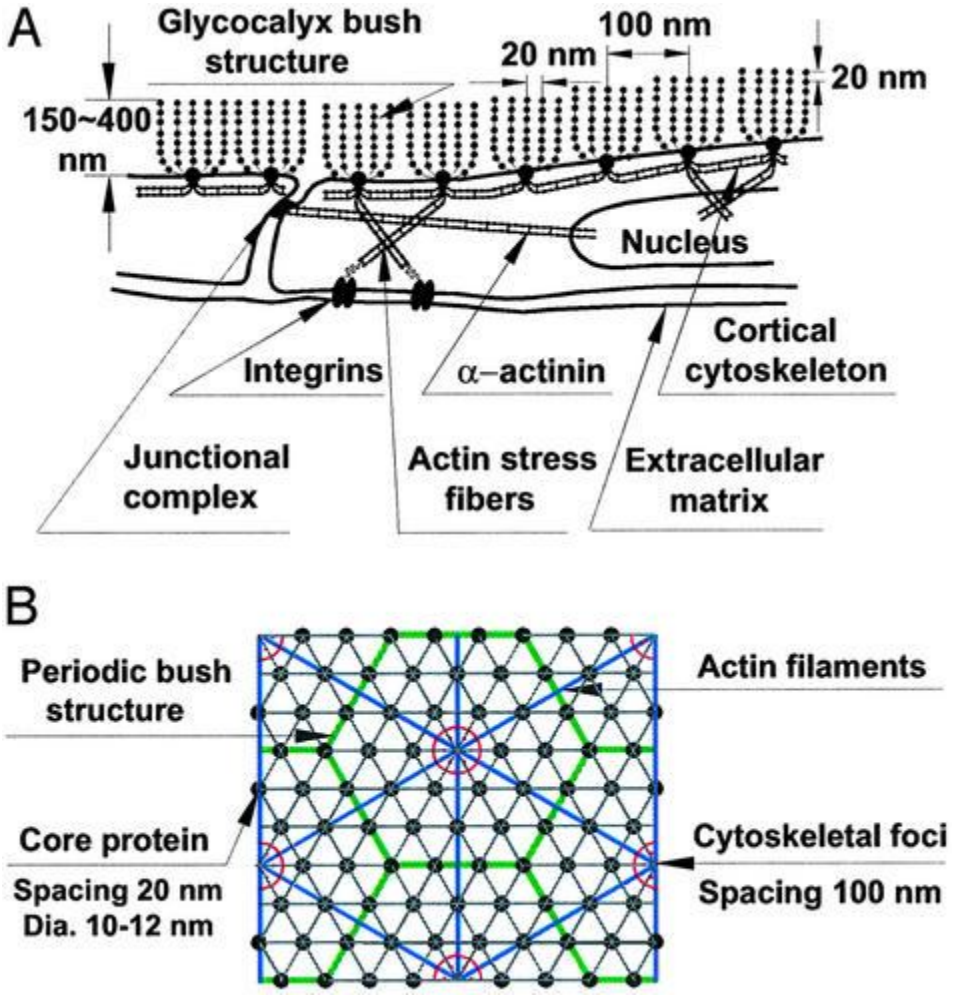
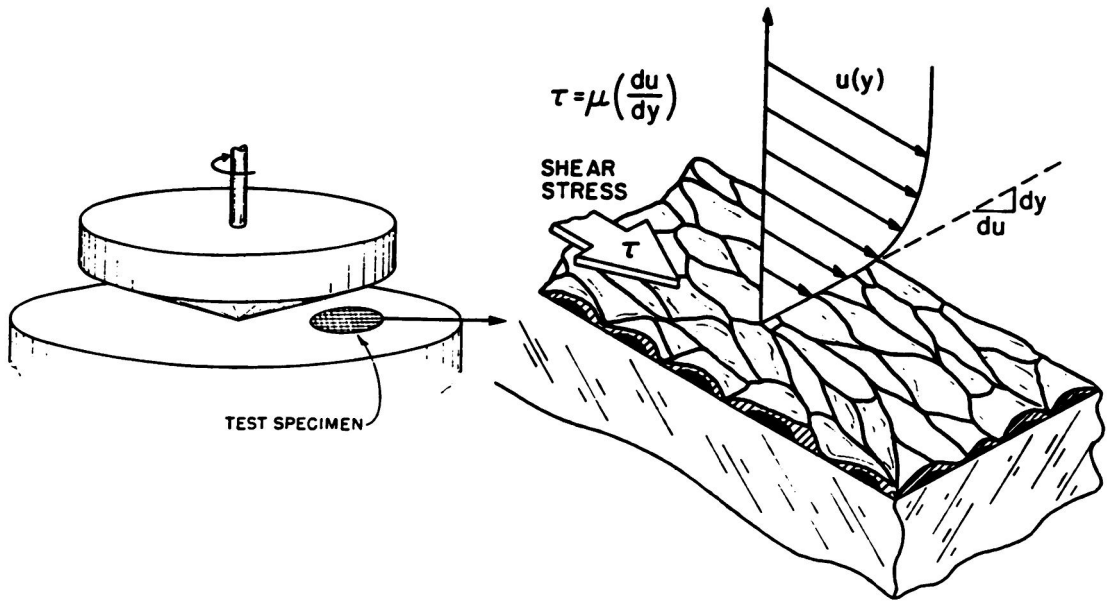


Fig.1.10. (*A*) Sketch of ESL (not to scale) showing core protein arrangement and spacing of scattering centers along core proteins and their relationship to actin CC. (*B*) *En face* view of idealized model for core protein clusters and cluster foci and their relationship to hexagonal actin lattice in CC (Weinbaum, S et al., 2003).

1.3.4 Flow Experiment Apparatus

The cone – plate flow system and parallel – plate flow chamber are widely used flow experiment apparatus to apply FSS on cultured cells. In 1981, an in vitro system called cone-plate flow system was developed by Dewey and his colleagues to study shear stress effects on endothelial cells. A rotating cone applies fluid shear stress to endothelial cell monolayers maintained on glass coverslips. The shear stress force (τ) depends upon the fluid viscosity and the detailed distribution of flow velocities near the cell surface as shown schematicall in fig 1.11a. A cone plate has stationary flat plate and a rotating cone. Since both the distance between the cone and plate surfaces and the local relative velocity increases linearly with the distance from the cone axis, this configuration provides constant shear stress regardless of position. Depending on the taper and the imposed angular velocity, a wide range of shear stresses can be applied to the attached cells (Ohashi, T and Sato, M 2005)

a)



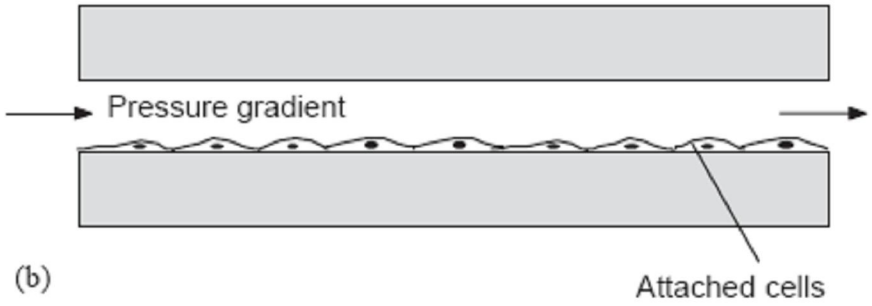


Fig.1.11. Flow experiment apparatus a) cone –plate flow chamber, (Davies PF et al., 1984), b) Parallel plate flow chamber (Ohashi, T and Sato, M 2005)

Cells can be seeded on coverslipes and placed on stationary flat plate. The cone's rotational speed can be adjusted to produce laminar FSS between 0.01 and 100 dyne/cm^2 (Thanawut, T., 2003).

Another is a parallel – plate flow chamber that provide a controlled environment for determinations of the shear stress at which cells in suspension can bind to endothelial cell monolayers (Munn, LL et al., 1994). Fluid flow causes laminar flow between two plates by an pressure gradient at either end of flow chamber figure. 1.11(b). After cells are seeded onto a coverslipe, it is placed to the bottom surface of the flow chamber. The flow circuit consists of the flow chamber, a reservoir, a roller pump, and a damping chamber (Ohashi,T and Sato,M 2005).

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Equipments

Class II Laminar Flow Hood

Thermo electron corporation hepa class 100, % 5 CO_2, 37 °C huminified incubator

Cole Parmer Instrument Company Masterflex L / S 0.1 ml/min-3460ml/min pump

Carl Zeiss 426126 Fluorescence Microscope

Olympus CK2 Inverted microscope

A620 Canon camera

A parallel plate flow chamber (original)

Tygon hoses 15 and 25 cm, MASTERFLEX

75 cm^2 tissue culture flasks GREINER

Centrifuge machine universal 32R

Water Bath GFL

Sterile serologic pipettes 2 ml, 5 ml, 10 ml, GREINER

Falcon tubes 15 ml and 50 ml, GREINER

Quadriperm plate, GREINER

2.1.2 Chemicals

Gelatin 500g.	:	APPLICHEM. A-1693 DATEKS
(DMEM) with L-glutamine 500 ml	:	BIOCHROM
PBS (1x) solution 500 ml	:	BIOCHROM
(FBS) 500 ml	:	BIOCHROM
Trypsin 0.25% EDTA, 500 ml	:	INVITROGEN
Penicillin Streptomycin Sol, 20ml	:	INVITROGEN
VE-Cadherin antibody 100 µg/ml	:	CHEMICON
GAM-IgG-Alexa (1mg/mL) (Mouse, red	:	SANTA CRUZ
Heparinase III from Flavobacterium heparinum	:	SIGMA
Chondrotinase ABC from Proteus vulgaris	:	SIGMA

2.2. METHODS

2.2.1 Shear Stress Equation between Parallel Flow Chamber

From fluid mechanics, for the case of parallel plates, the following assumptions are made;

- Newtonian fluid; presence of cells is neglected
- Fully developed flow
- Incompressible fluid
- No-slip boundary condition
- 2-D flow

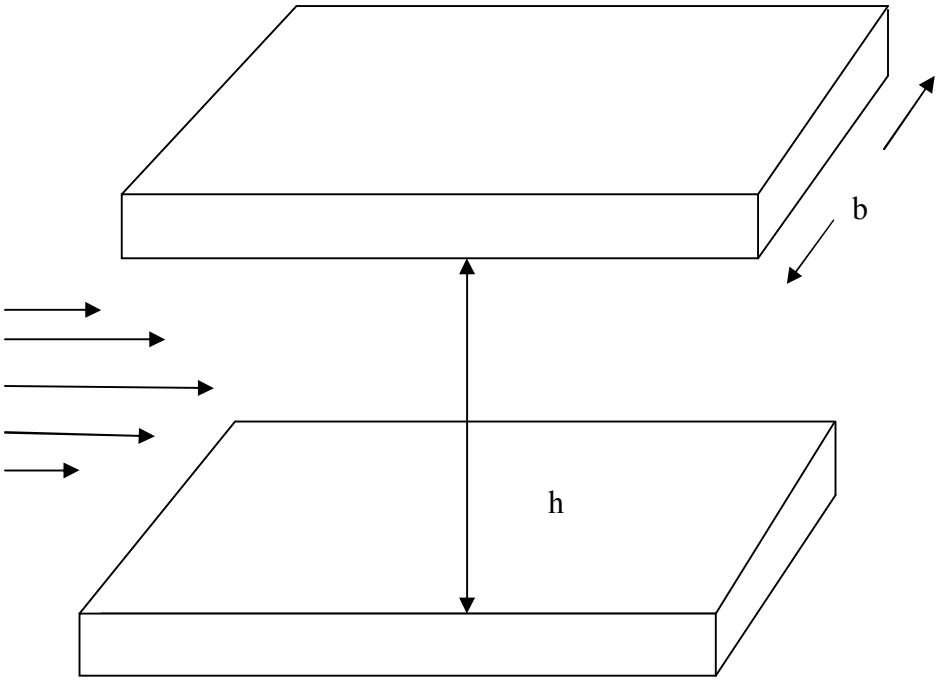


Figure. 2.1 Labelled paralel – plate flow chamber

$$\frac{\partial^2 u}{\partial y^2} = \frac{1}{u}\frac{\partial p}{\partial x} \quad (1)$$

$$\frac{du}{d\mu} = \left[\frac{1}{\mu}\frac{\partial p}{\partial x}\right] y + C_1 \quad (2)$$

$$\tau = \mu \frac{du}{dy} \quad (3)$$

$$\tau = \mu \left(\frac{1}{u}\frac{\partial p}{\partial x} y + C_1\right) \quad (4)$$

$$\tau = h\left(\frac{\partial P}{\partial x}\right)\left(\frac{y}{h} - 2\right) \quad (5)$$

$$Q = b - \frac{1}{12u}\left(\frac{dp}{dx}\right) h^3 \quad (6)$$

$$\tau_{wall} = \frac{6Q\mu}{bh^2} \quad (7)$$

Equation 1 is integrated to equition 2. Shear stress (τ) is propotional to the dynamic viscosity (μ) of the fluid flow (Roluleau, L, 2006). By the definition of a Newtonian Fluid we know that equation 2 is proportional to shear stress on the walls of the parallel plate. Combination of 2 and 3 equitions, we can get equition 5. Q is the volumetric flow, equition 5 can be written as equition 7. In the equition 7, b is the channel widht, h is the height of the chamber.

With these parameters, we also find Reynold number (Re) with entrance lenght L, that generate a flow from disturbed to steady across an appropriate canal length.

$$\frac{L}{h} \cong 0.06\, Re = 0.06\frac{\rho V h}{\mu} = 0.06\frac{\rho Q}{b\mu}$$

Re = ρVh/μ, ρ is the density of the flow, as mention below μ is the viscosity of the flow, V is the velocity of the flow and h is the distance between the top and the bottom of the flow chamber. b is the channel weight.

2.2.2 Cell Culture

We used Human Umbilical Vein Endothelial cells (HUVECs) in all our experiments. They were obtained from GATA (Gulhane Military Medicine Academy, Hematology department) and stored at nitrogen tanks (-80°C). HUVEC are isolated from normal human umbilical vein. They are cryopreserved at the end of primary culture and can be cultured and propagated at least 16 population doublings. HUVEC are responsive to cytokine stimulation in the expression of cell adhesion molecules. These cell systems are commonly used for physiological and pharmacological investigations, such as macromolecule transport, blood coagulation, and fibrinolysis.

HUVECs were cultured in DMEM medium supplemented with 10% (FBS) and 1% penicillin – streptomycin.

Gelatin preparation; 0.1 gr powder gelatin is weighed by a sensitive scale device and added 50 ml falcon tube. After that, powder gelatin is resuspended in 50 ml distilled water in 50 °C water bath. Dissolved gelatin is filtered by syring filter into a sterile bottle in the flow hood

- Before cell seeding, 75cm^2 tissue culture flasks coated with 0.2% Gelatin for 1 hrs.
- Aspirate gelatin and then aspirate medium of HUVECs from flask
- Wash with PBS for eliminate the dead cells
- Add tyripsin and wait 2-3 min, check the cells under microscope to be sure they detached from monolayer surface.
- Add DMEM on tyripsined cells, take all to a 15 ml falcon tube and then centrifuge at 2500 rpm, for 5 min at 27°C.
- Discard supernatant and resuspend in DMEM
- After counting cells (hemocytometer measurements), cell cultures were kept in a 5% CO_2 humidified incubator at 37°C

2.2.3 Flow Chamber Design

A parallel plate flow chamber was originally designed at our laboratory and used to expose cultured HUVEC's monolayers to fluid shear stresses Figure 2.2.

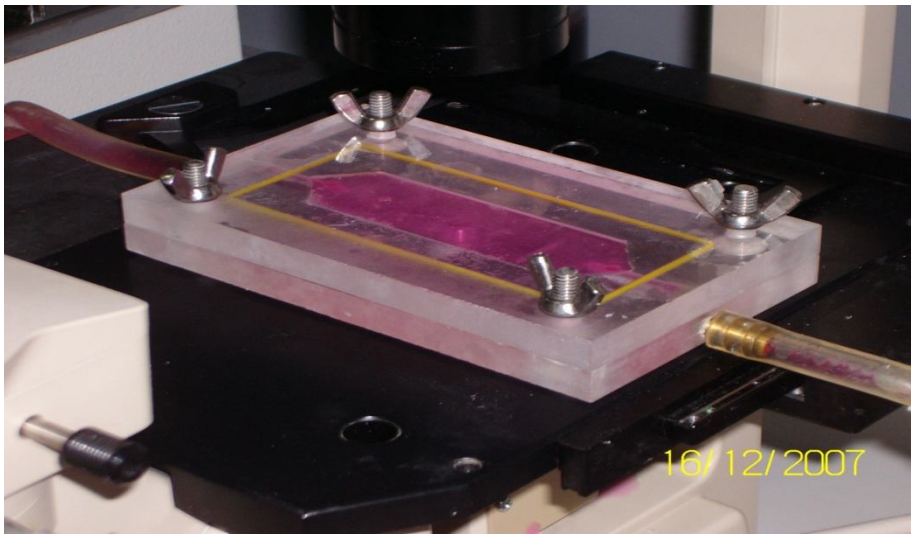


Figure 2.2 Picture of parallel–plate flow chamber

Parallel–plate flow chamber was made from Plexiglas material Figure 2.3. 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 a channel which has the width 7.5 cm. and 0.2 cm depth. The channel is insulated by and O-ring to prevent the DMEM flow leakage from channel to outside. The entrance length of the channel is almost 5 cm.

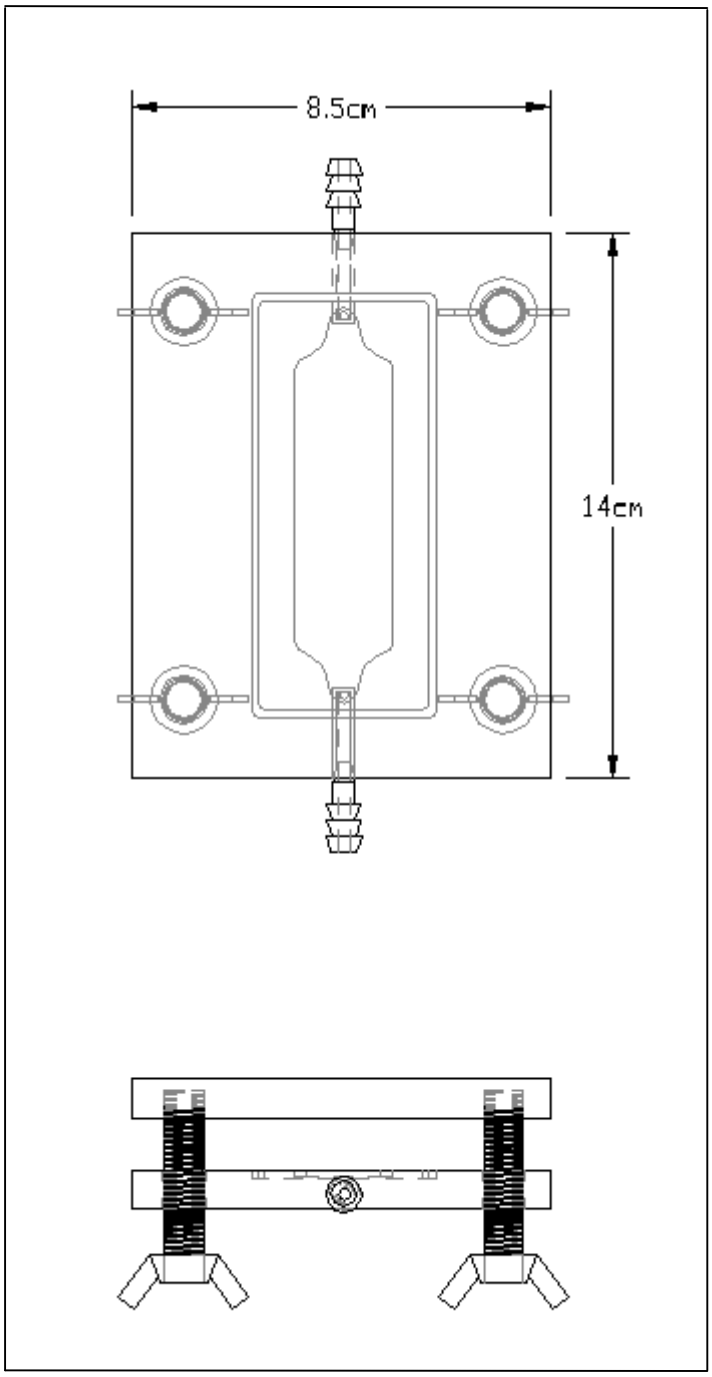


Figure 2.3 Technical drawing of parallel plate flow chamber

2.2.4 Flow System Setup

Flow system is composed of a flow chamber, two DMEM medium bottles supplemented with 10% FBS and 1% penicillin-streptomycin (one of them is used as reservoir, other one is used as dampener), a pump, hose (Tygon-15cm), water bath, and inverted microscope shown in Figure 2.4.

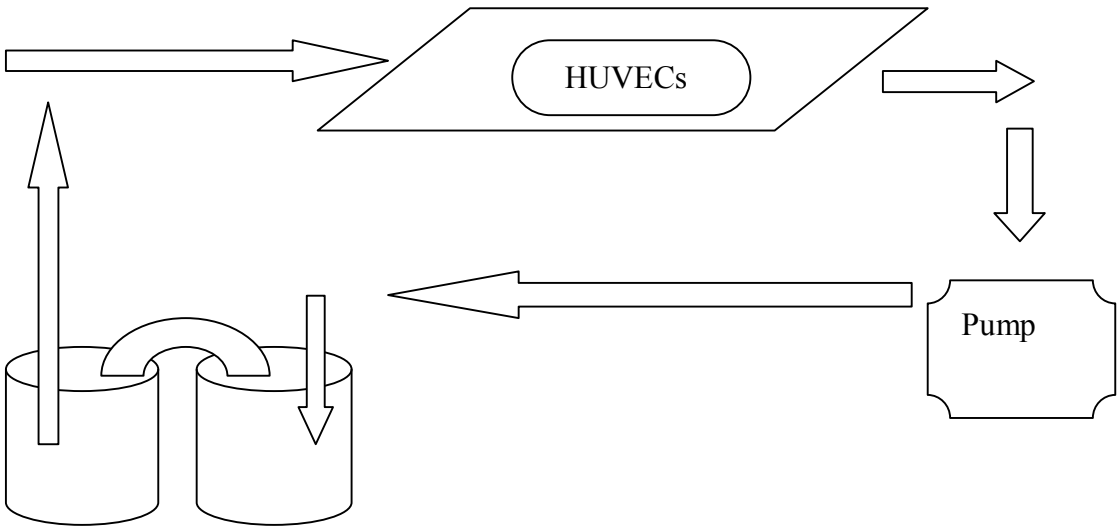


Figure 2.4 Schematic appearance of flow system

The pump provides us to generate steady flow pushing 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 leakage. The purpose of using two DMEM bottle is to prevent bubble formation. During the experiments, the system was kept at 37^0C in the water bath. A 25-75 mm coverslide was seeded with HUVECs in Quadriperm plate (GREINER) which were cultured until reaching a confluent monolayer (2-3 days) and transferred to flow chamber gently for experiment, Figure 2.5. Morphological changes were observed by inverted microscope. Then experiment images were taken by a fluorescence microscope camera.

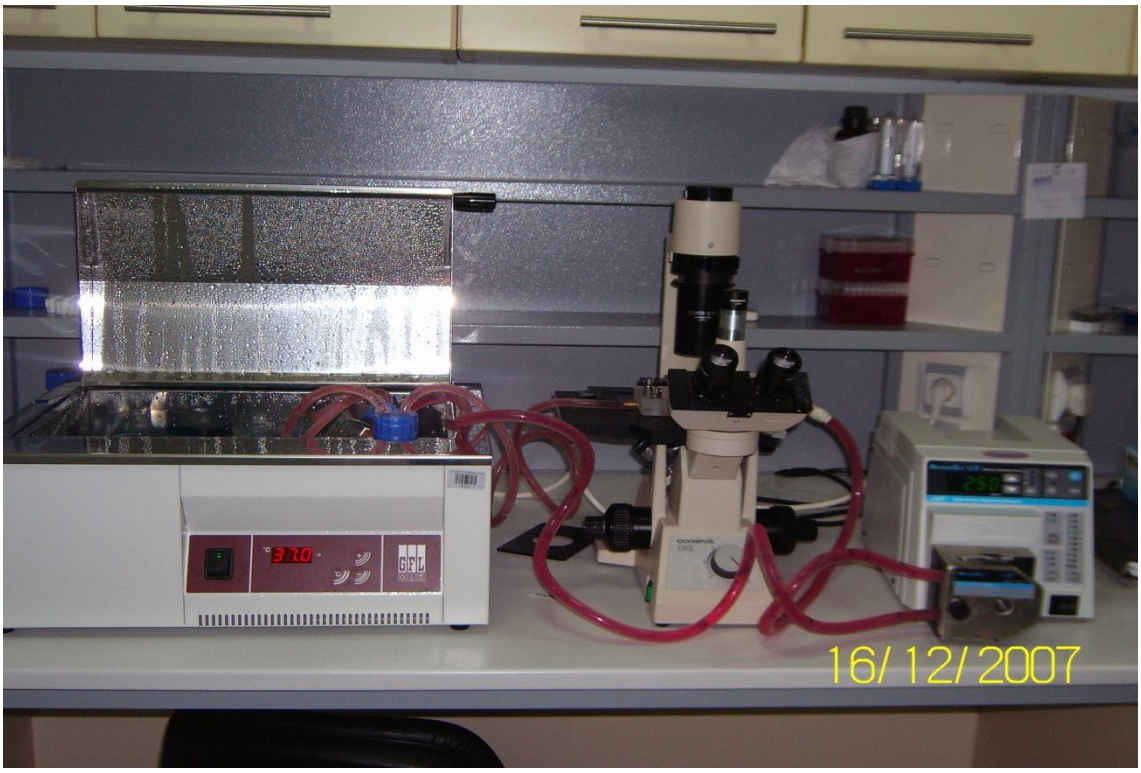


Figure.2.5 Picture of the flow system

2.2.5 Sterilization

Before starting flow experiments, flow chamber, hoses, coverslips and Quadriperm plates were sterilized. We used autoclave for sterilization of hoses, distilled water and coverslips. Flow chamber was waited in 70% ethanol for 30 min and then washed with sterilized water under hood and set to dry. For an effective sterilization UV lamp was used.

2.2.6 Immunofluorescense

2.2.6.1 Detection of VE-Cadherin

1.10^5 cells / well were seeded on two well of the 24-well plate for detection of surface antigen VE-Cadherin. 1st Ab mixture include 30 µl VE-Cadherin (100 µg/ml, Chemicon) in (20µg/ ml) and 120 µl DMEM + % 10 FBS was added 55µl each well but , VE-Cadherin Ab should be sterilized by filtration to maintain the cultures after staining. Cells were incubated at 5 % CO_2, 37°C, for 30 min. After removing Ab solution cells were washed with fresh medium three times. 2nd Ab mixture include 1.2 µl GAM-IgG-Alexa (1mg/mL)(Mouse, red) and 118 µl DMEM+10% FBS was added 55µl each well in dark. Incubated at 5% CO_2, 37°C, for 30 min, washed with PBS three times. Cells were visualized under PBS (with Ca and Mg) using fluoresce microscope. The cells are still alive at this point, so they can be recultured if necessary. Alternatively, the cells can be fixed by treatment with a 4% PFA solution for 20 min. (500µL/well) and then stored under a 50:50 solution of PBS: glycerol

2.2.7 Degragation of Glycocalyx

2.2.7.1 Heparinase Treatment

Heparinase is an enzyme used for degradation of various heparin substrates. This enzyme can be reconstituted in 20 mM Tris-HCl, pH 7.5, containing 0.1mg/ml BSA and 4 mM $CaCl_2$. Before the heparinase treatment, the cell monolayers were washed with serum free DMEM and then 200mU heparinase solution were placed on the HUVEC monolayers and incubated in 5% CO_2, 37°C for 30 min. After incubation, HUVEC monolayers were washed with PBS twice and then placed on the parallel plate flow chamber.

2.2.7.2 Chondrotinase ABC Treatment

Chondrotinase ABC catalyzes the eliminative degradation of chondrotin sulfate GAGs. Enzyme can be reconstituted in a 0.01% bovine serum albumin aqueous solution. Subsequent dilutions can be made into a buffer containing 50 mM Tris, pH 8.0, with 60 mM sodium acetate and 0.02% bovine serum albumin. Solutions should be prepared fresh. Before the Chondrotinase treatment, the cell monolayers were washed with serum free DMEM and

then 200mU heparinase solution were placed on the HUVEC monolayers and incubated in 5% CO_2, 37°C for 30 min. After incubation, HUVEC monolayers were washed with PBS twice and then placed on the parallel plate flow chamber.

CHAPTER 3

RESULTS

3.1. Steady Flow Experiments

Steady flow experiments are done under various flow rates considering the flow rates inside different cardiovascular regions such as coronary arteries, carotid arteries, etc. Three locations over the coverslips are considered, Figure 3.1. These are named as upper, middle and down regions.

To investigate the effect of laminar shear stress on EC morphology, HUVECs were exposed to 1000 ml/min, 1500 ml/min flow rates for 24 hours

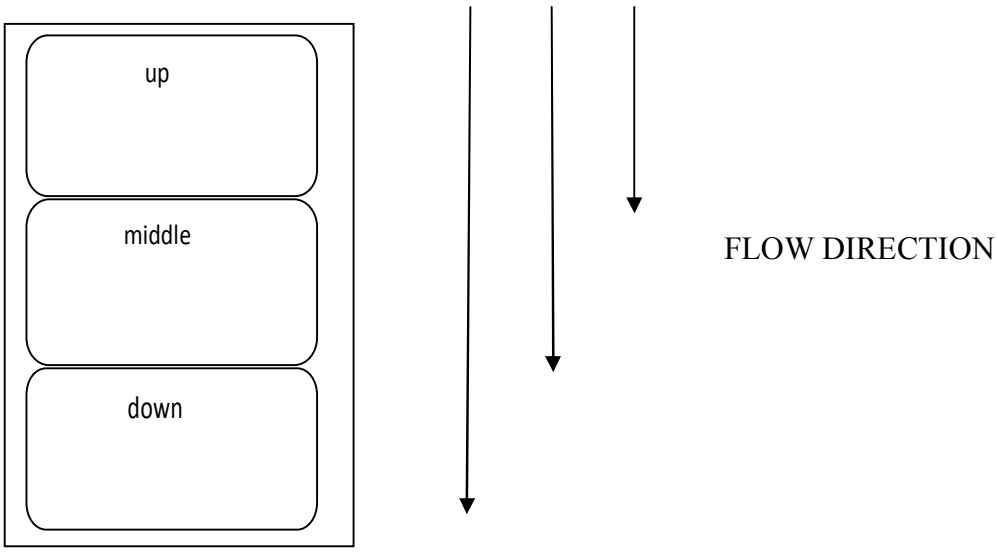


Figure 3.1 Schematic of 25-75 mm coverslips

Shear stress at the wall can be calculated for all experiments with following equation which is the characteristic shear stress calculation of the flow between parallel plates

$$\tau_{wall} = \frac{6Q\mu}{bh^2}$$

Q= 1000 ml/min

μ = 1 cP

b = 8.5 cm

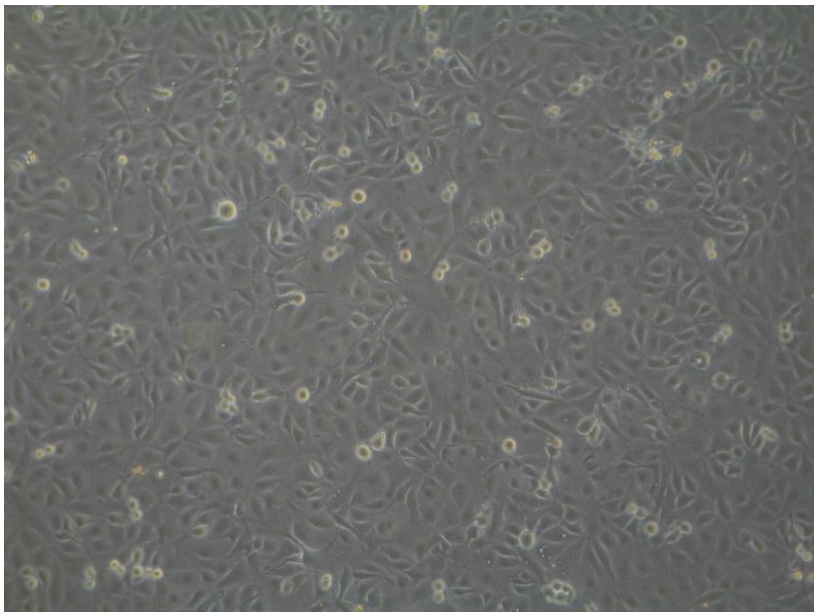
h=0.2

By applying a 1000 ml/min flow rate, we get a shear stress of 28 dynes/cm^2 at our first experiment.

3.1.1. Q=1000 ml/min 24 hour Steady Flow Experiment

Flow experiments are done inside the parallel flow chamber over the coverslips under the flow rate of 1000 ml/min during twenty four hours time period. The results of the experiment are given at three different locations. The morphology of the cells over the coverslips before the experiment are given in Figure 3.2-A, 3.3-A, and 3.4-A. It was shown that the cells have polygonal shape and confluent monolayer before the flow. HUVECs seeded on coverslips are aligned in the direction of the flow by exposed to high shear stress flow. Cell alignment through flow direction was observed in the upper part Figure 3.2-B, of coverslips which are more remarkable than middle 3.3-B and 3.4-B down parts.

A)



B)

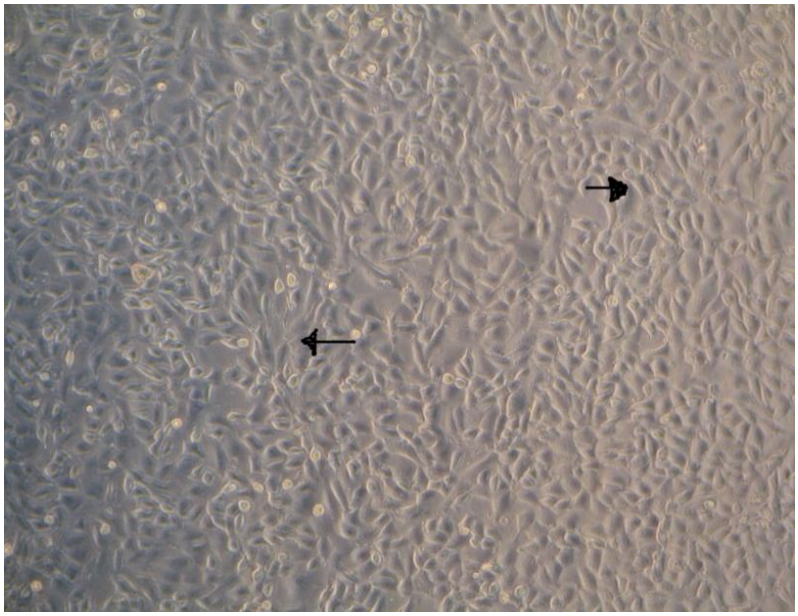
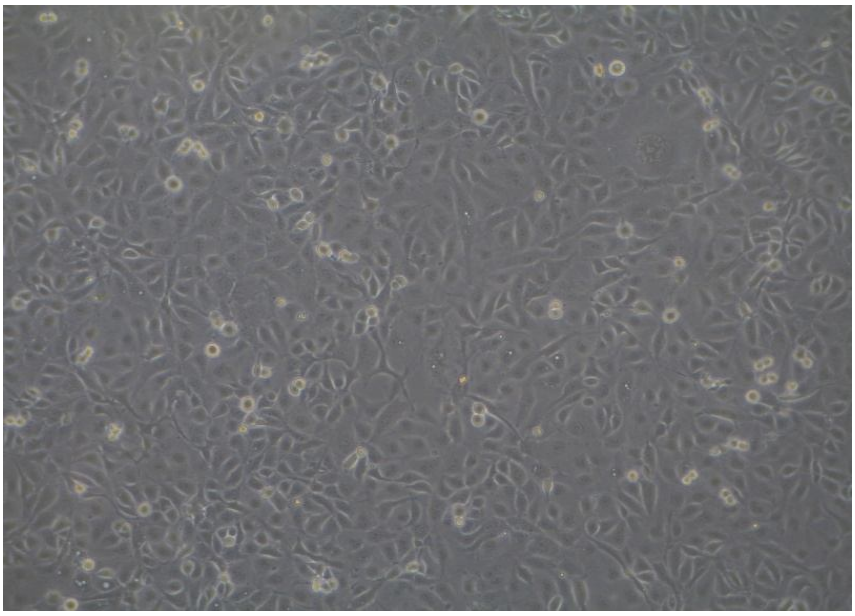


Figure 3.2 A) Morphology of HUVECs before 24 hour steady flow experiment upper region 10X. Cells have a polygonal shape and confluent monolayer. **B)** Morphology of HUVECs after 24 hour steady flow experiment upper region 10X. Arrows indicate the cell elongation along the flow direction. In these regions cell shape changed from polygonal to elipsodial

A)



B)

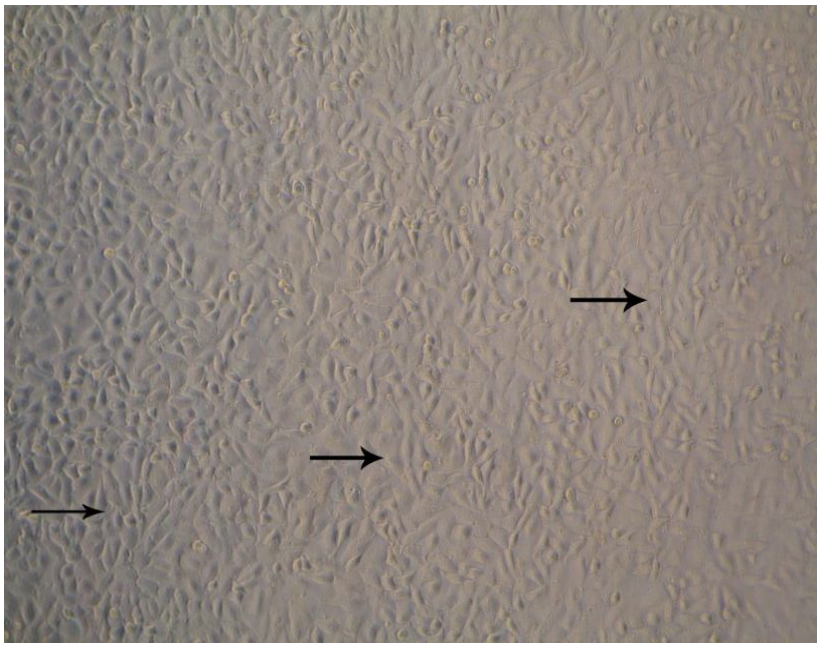
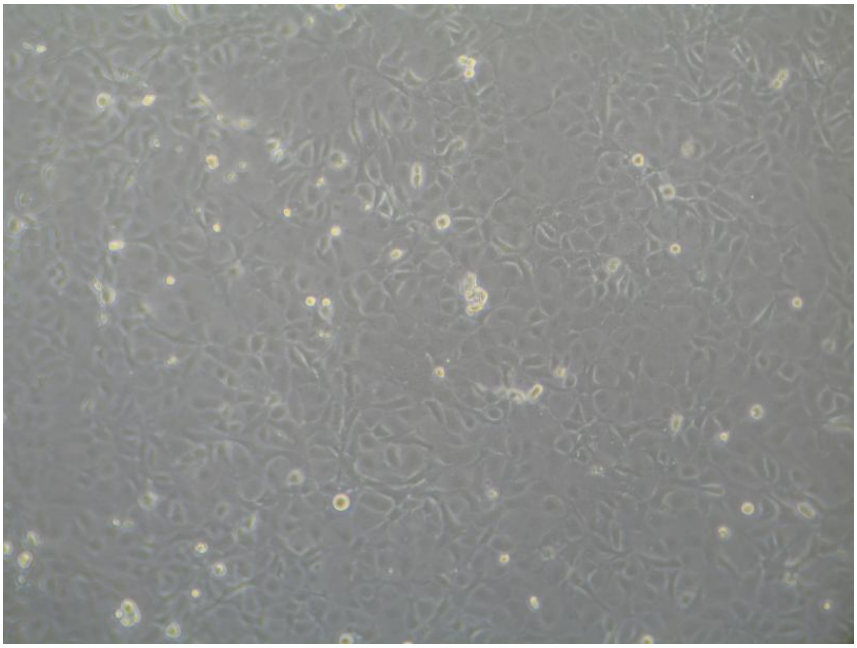


Figure 3.3 A) Morphology of HUVECs before 24 hr flow experiment middle region 10X. Cells have a confluent monolayer. **B)** Morphology of HUVECs after 24 hour steady flow experiment middle 10X. Cell alignment was shown on regions that arrows indicate.

A)



B)

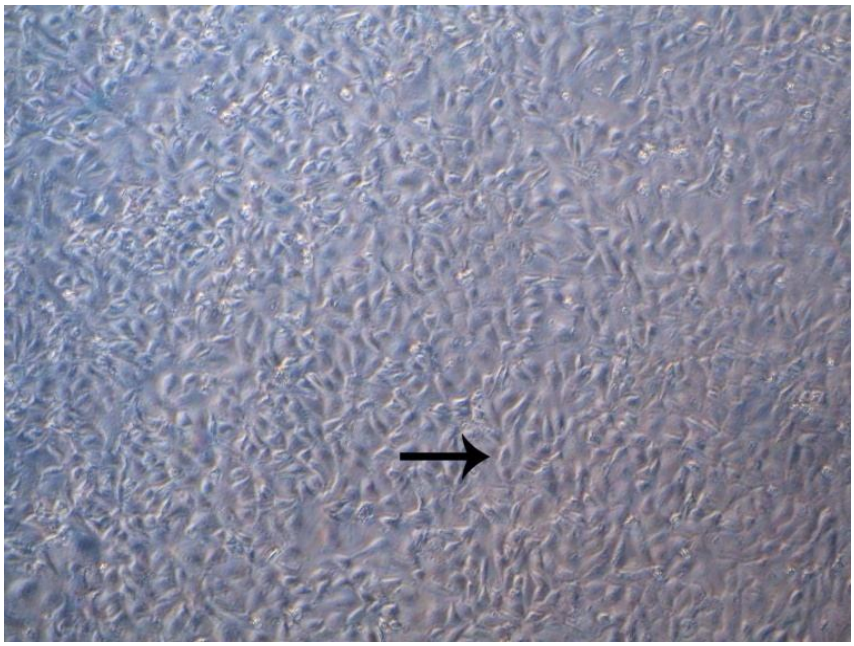
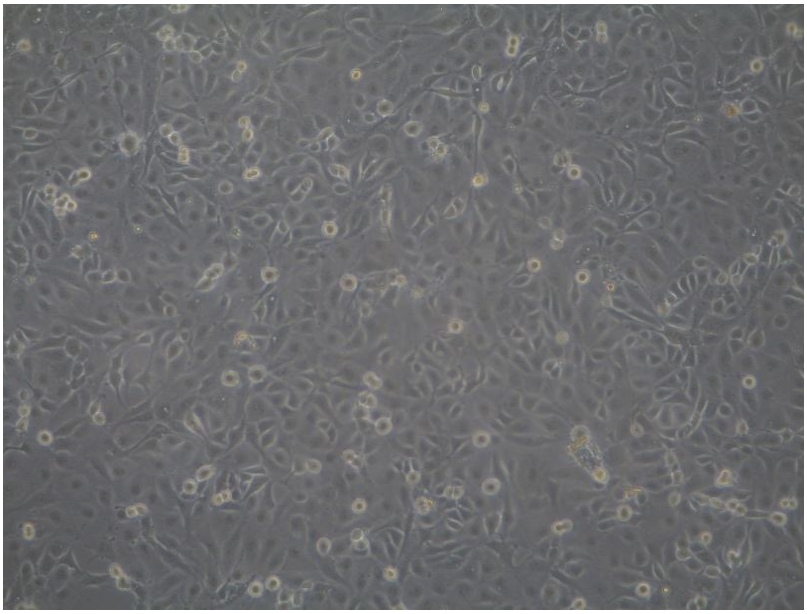


Figure 3.4 A) Morphology of HUVECs before 24 hour flow experiment down region10X. **B)** Morphology of HUVECs after 24 hour flow experiment down region 10X. Cell elongation is less remarkable than figure 3.2B and 3.3B.

3.1.2. Q = 1500 ml/min 24 hour Steady Flow Experiment

Flow experiments were done inside the parallel flow chamber over the coverslips under the flow rate of 1500 ml/min during 24 hours time of period. Shear stress exposed to cells is 42 dyne/cm^2. In this experiment coverslips were separated three regions as it was done in the first experiment. Figure 3.5-A and 3.6-A show the morphology of HUVECs before experiment. In figure 3.5-B and 3.6-B regional gaps and regional cell elongation were observed

A)



B)

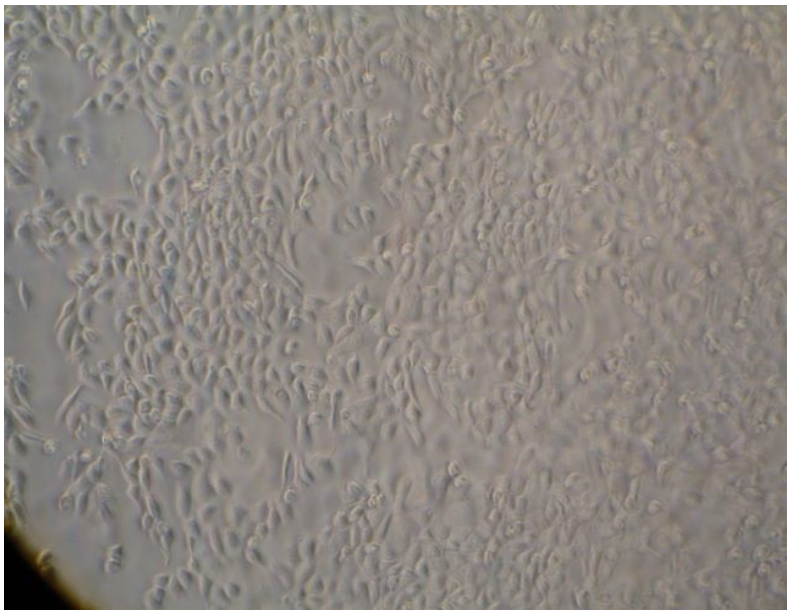
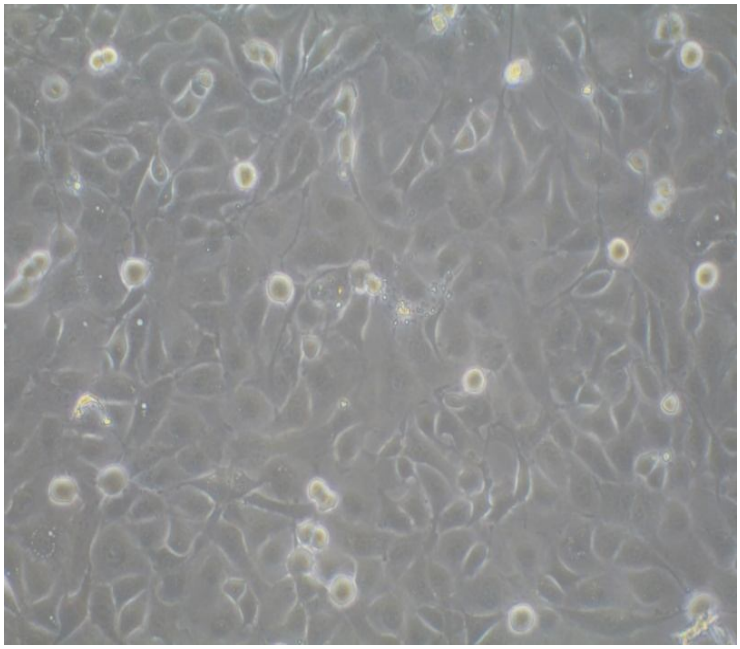


Figure 3.5 A) Morphology of HUVECs before 24 hour flow experiment upper region 10X, Cells are polygonal and confluent, **B)** Morphology of HUVECs after 24 hour flow experiment upper region 10X. Regional gaps were formed by high shear stress, some morphological changes from polygonal to elipsodial were observed. Regional cell detachment was observed.

A)



B)

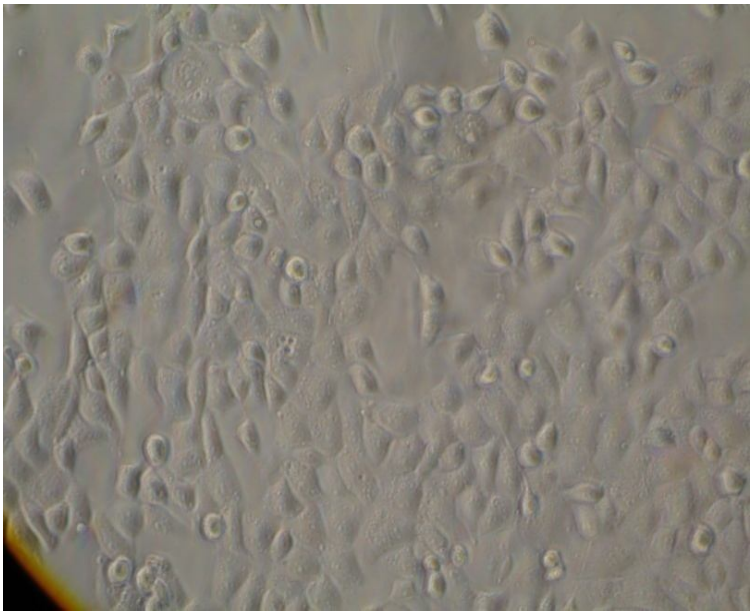


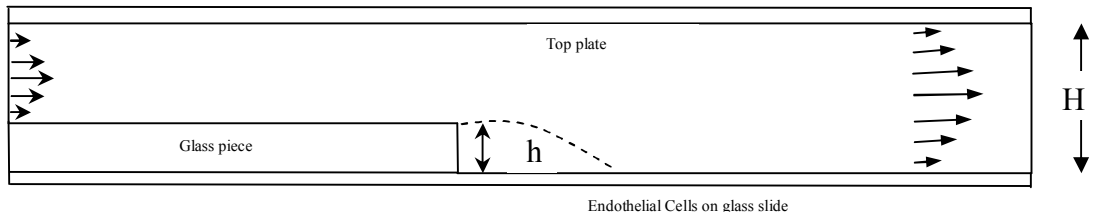
Figure 3.6 A) Morphology of HUVECs before 24 hr flow experiment middle region 20X. **B)** Morphology of HUVECs after 24 hr flow experiment middle region 20X. Cells in the left part of the picture, aligned in the direction of the flow.

3.2 Disturbed Flow Experiments (Step Flow Experiments)

Atherosclerotic plaques localize at curvatures and branches of the arterial system, where the flow is often disturbed as shown in the Figure 1.2. Disturbed laminar flow, characterized by areas with reversed flow (i.e., flow separation, recirculation, and reattachment or stagnation point).

There is a setup established to simulate the disturbed flow as shown in Figure 3.7. Two coverslips were sticked to each others. One of the coverslipe thickness was 1 mm and the other one was 0.1 mm as shown figure 3.7a. HUVEC was seeded on top of the bottom coverslips. Then the flow was allowed over the cells. The computational sample calculation (CFD) is shown by Figure 3.7b, the flow through the channel for step flow experiment. This figure shows the stagnation and also separation points for the experiment. Depending on the flow rate the location of the stagnation point is changed Figure 3.7 b.

a)



b)

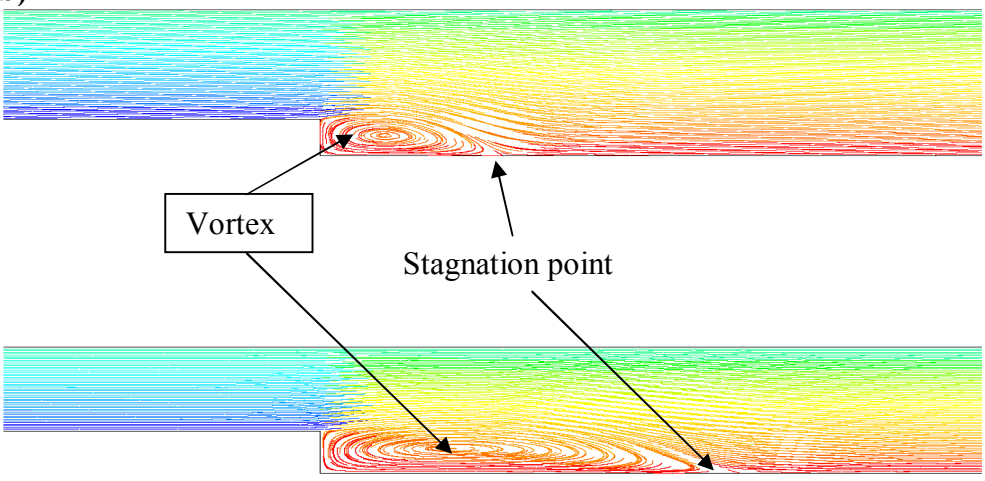


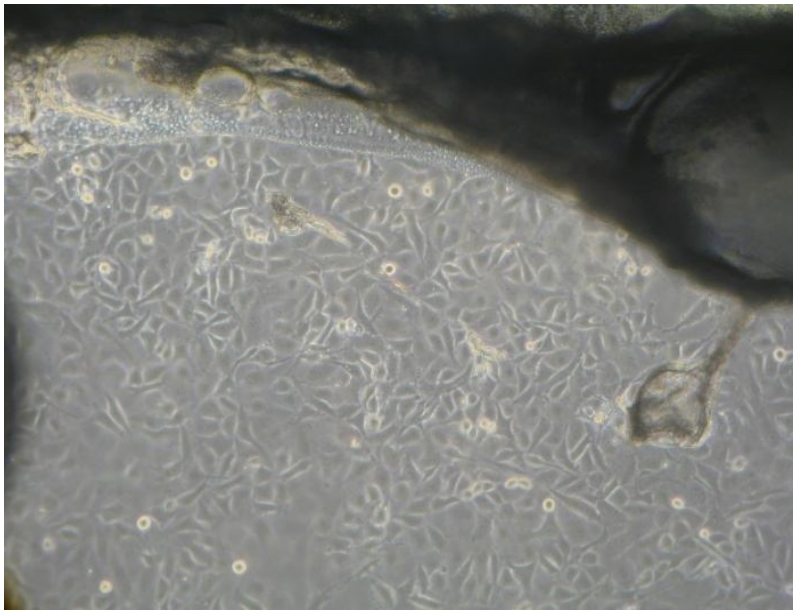
Figure 3.7 a) Schematically presentation of step flow apparatus **b)** CFD simulation model of apparatus.

The ratio of h/H is 0.1/1=0.1. HUVECs were exposed to disturbed flow at 250 ml/min during 5 hour, 500 ml/min during 8 hours, and 150 ml/min during 10 hour.

3.2.1 Q = 250 ml/min, 5 hour Disturbed Flow Experiment

Step flow experiment was done inside parallel plate flow chamber and the flow that moved along the step flow model, gave us interesting results. Flow rate determined the reattachment site on the coverslips. Results before the experiment as shown in Figures 3.8-A, 3.9-A, 3.10-A, and 3.11-A, cells had confluent monolayer. Step is clearly seen in this up region in figure 3.8-A and 3.8-B. After 5 hours flow experiment, regional cell lost was observed on the stagnation point shown in figure 3.8-B, 3.9-B, at this area cells detached from the monolayer. In the figure 3.10-B, cell shape became rounded after experiment. Appearance of gaps in the cell monolayer is clearly shown in figure 3.11-B and cell shape was more polygonal in the down region than the middle region of the coverslips.

A)



B)

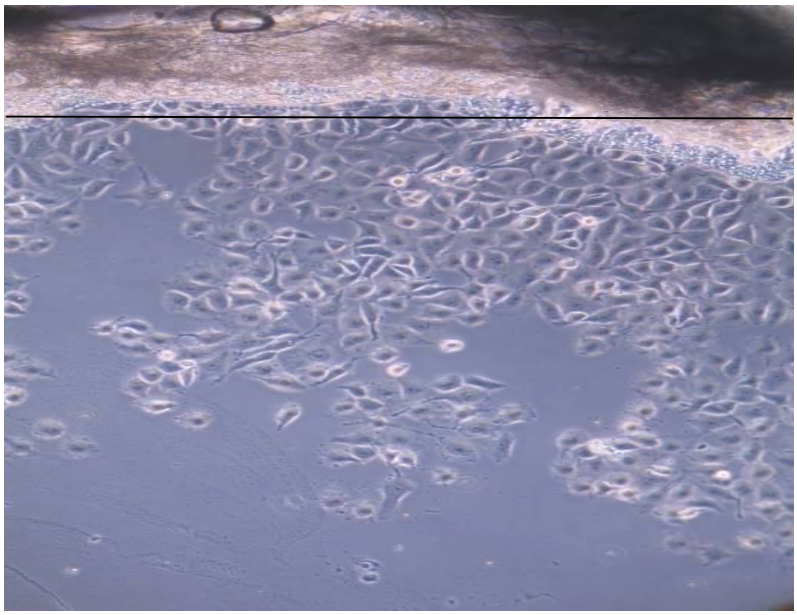
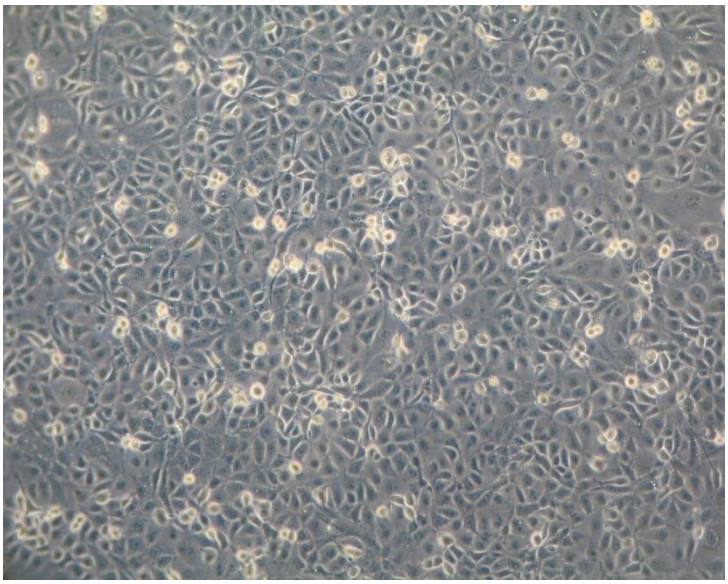


Figure 3.8 A) Morphology of HUVECs before 5 hour disturbed flow experiment upper region 10X. **B)** Morphology of HUVECs after 5 hour disturbed flow experiment up 10X, flow rate caused cell lost on the reattachment site. Some of the cells were not influenced from the flow effect and they were still alive. There was not a remarkable morphological change on HUVECs.

A)



B)

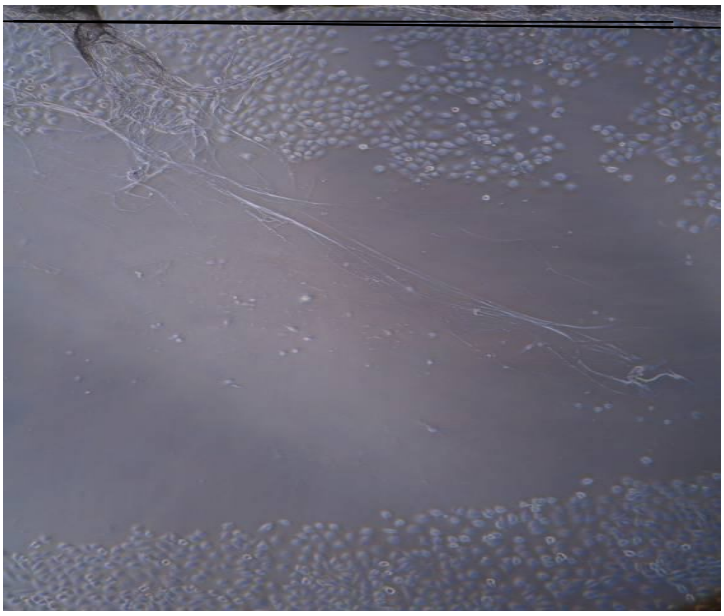
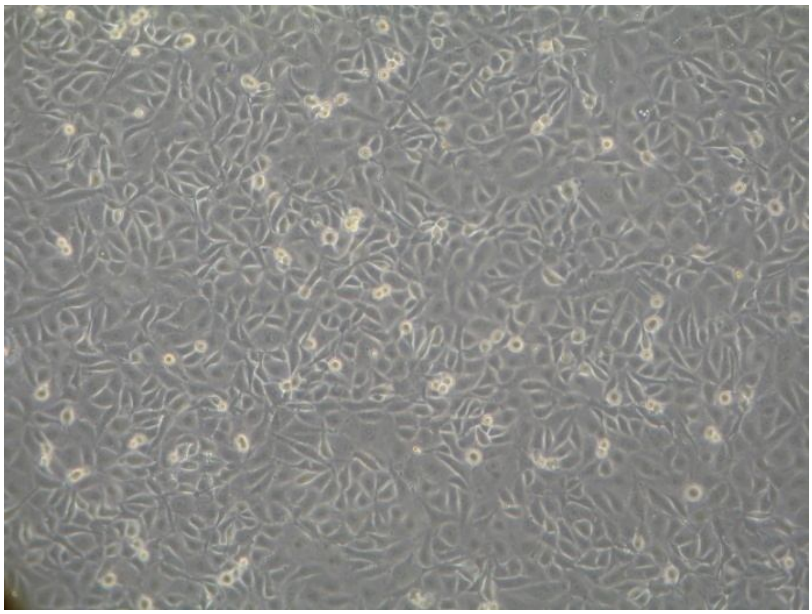


Figure 3.9 A) Morphology of HUVECs before 5 hour step flow experiment the downstream of step 10X. Cells have confluent monolayer. **B)** Morphology of HUVECs after step flow experiment downstream of step 5X. The region that flows fallen down generated a band lying down throughout the stagnation point. In this region cells detached from the monolayer.

A)



B)

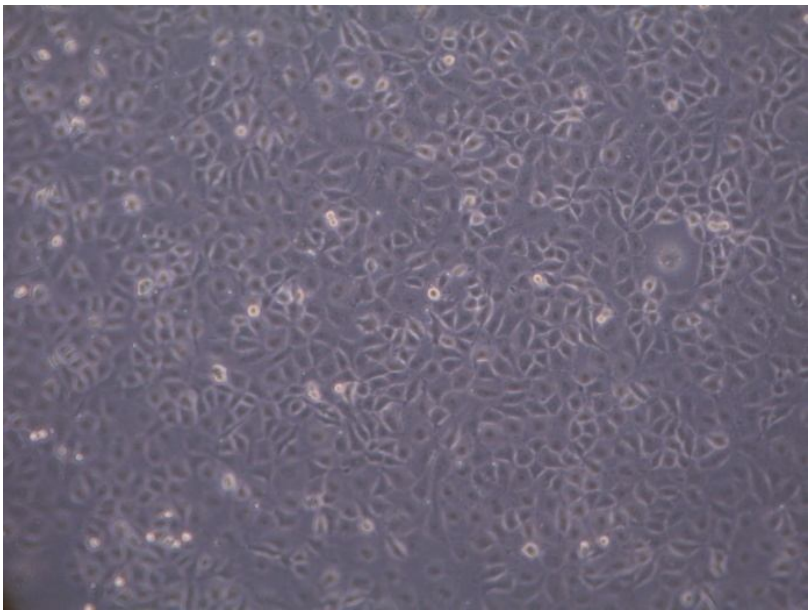
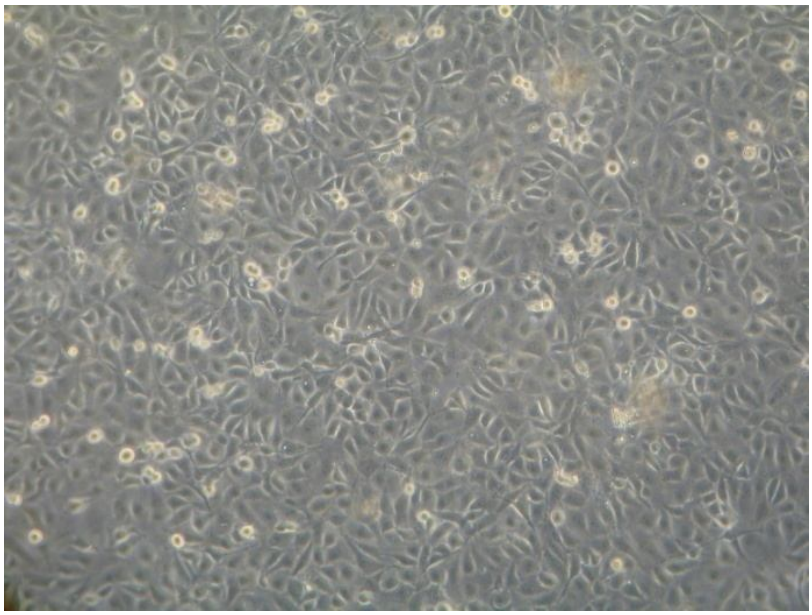


Figure 3.10 A) Morphology of HUVECs before 5 hour disturbed flow experiment middle region 10X. **B)** Morphology of HUVECs after 5 hour disturbed flow experiment middle region 10X. In this region cell morphology changed into more polygonal shape.

A)



B)

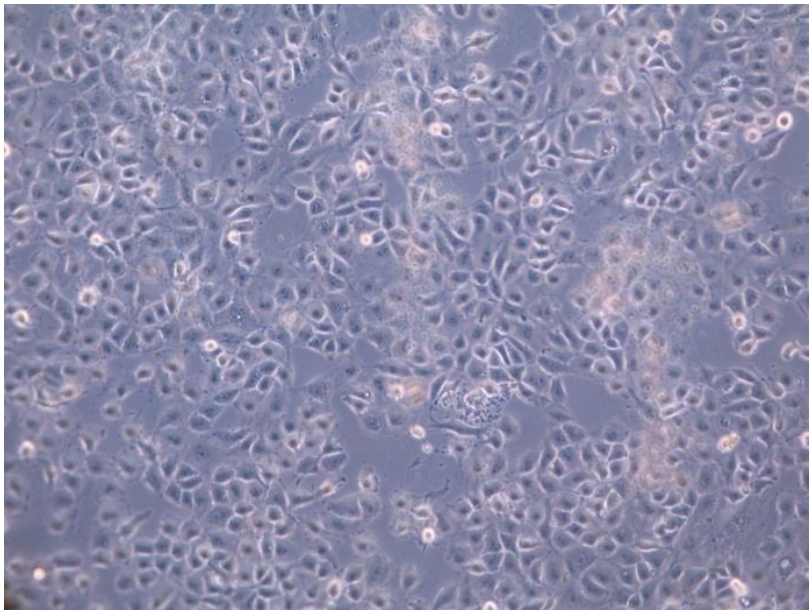
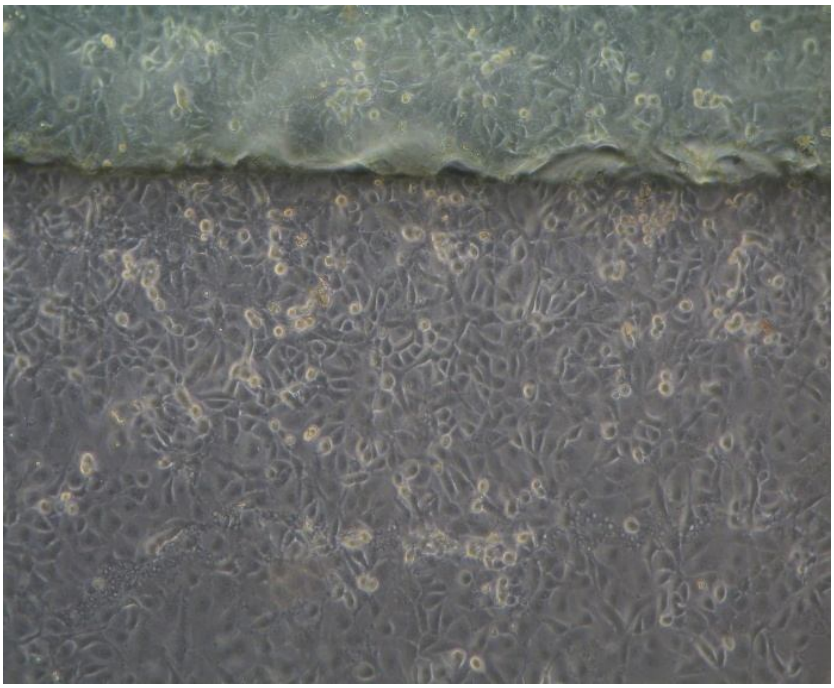


Figure 3.11 A) Morphology of HUVECs before 5 hour disturbed flow experiment down 10X. **B)** Morphology of HUVECs after 5 hrs disturbed flow experiment down 10X. There are gaps between cells and shape of the cells became more polygonal than middle region.

3.2.2 Q = 500 ml/min 8 hour Disturbed Flow Experiment

The step flow model allows the study of the responses of ECs to disturbed flow in the reattachment area and laminar flow regions. In this experiment the flow rate was increased to 500 ml/min that has a shear stress of 14 dynes/cm^2 and time period of 8 hours. Figures 3.12-A, 3.13-A, 3.14-A and 3.15-A shows the monolayers of the HUVECs in static culture before disturbed flow experiment. The flow at 500 ml/min flow rate caused the cell dead and cell lost after disturbed flow experiment, as shown in the figure 3.12-B. Also, Figure 3.14-B and 3.15-B show the cell elongation like band across the middle and down regions of the coverslips

A)



B)

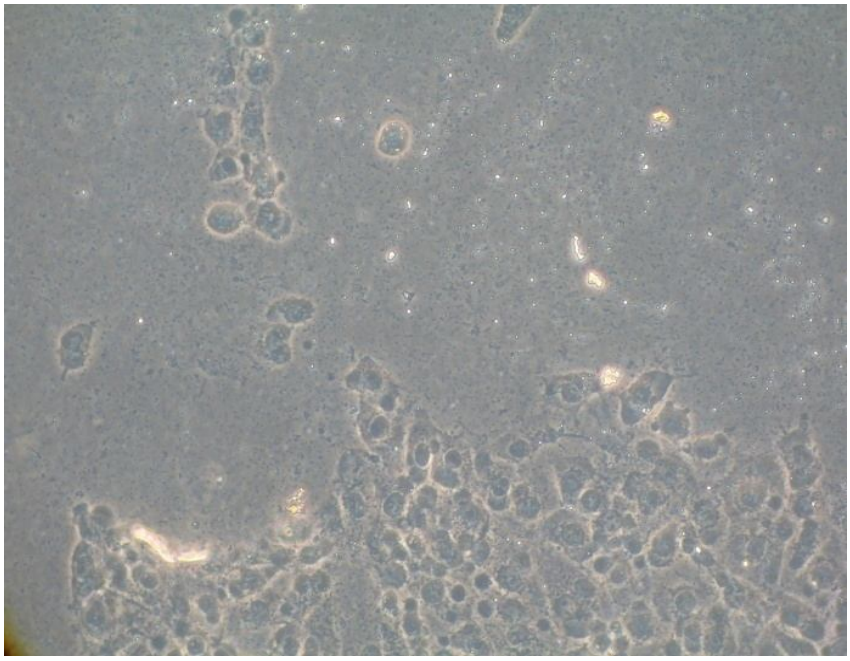
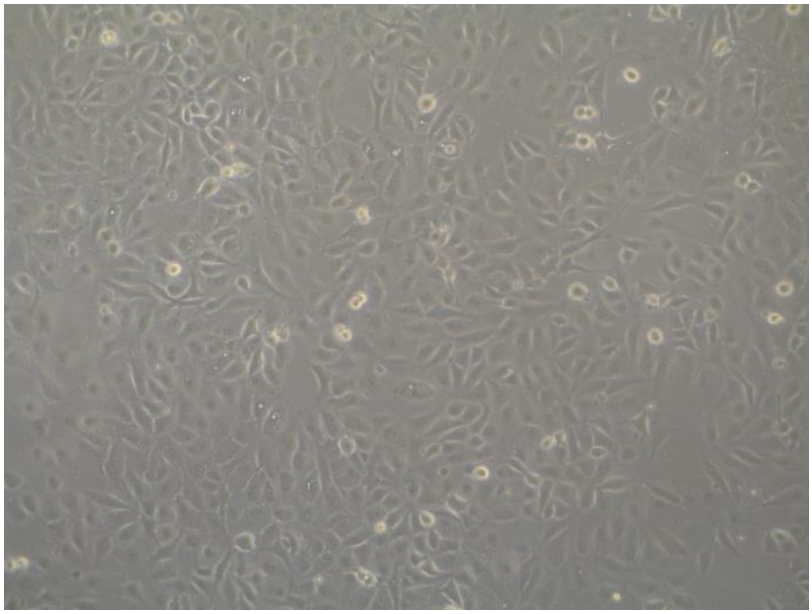


Figure 3.12 A) Morphology of HUVECs before 8 hrs disturbed flow experiment upper region 10X. **B)** Morphology of HUVECs after 8 hour disturbed flow experiment upper region 10X. Cell lost at the stagnation point and cell death in the downstream of stagnation point were observed.

A)



B)

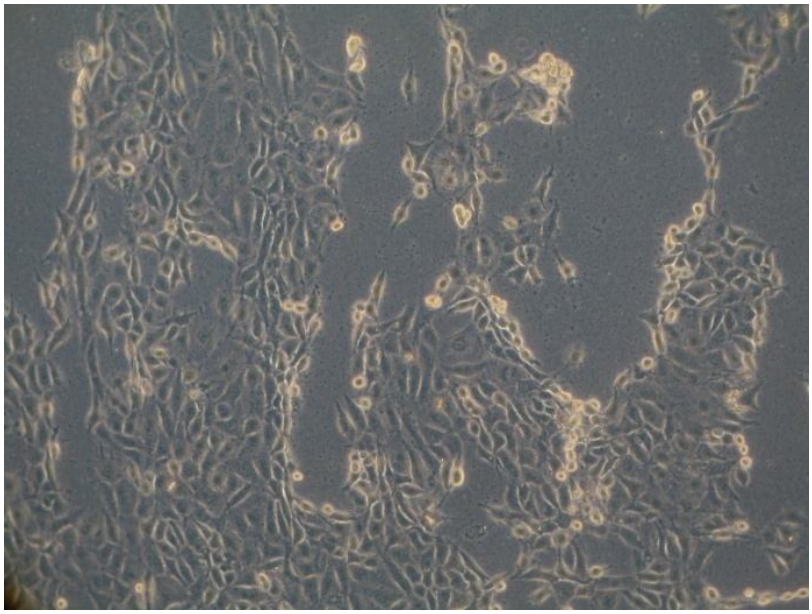
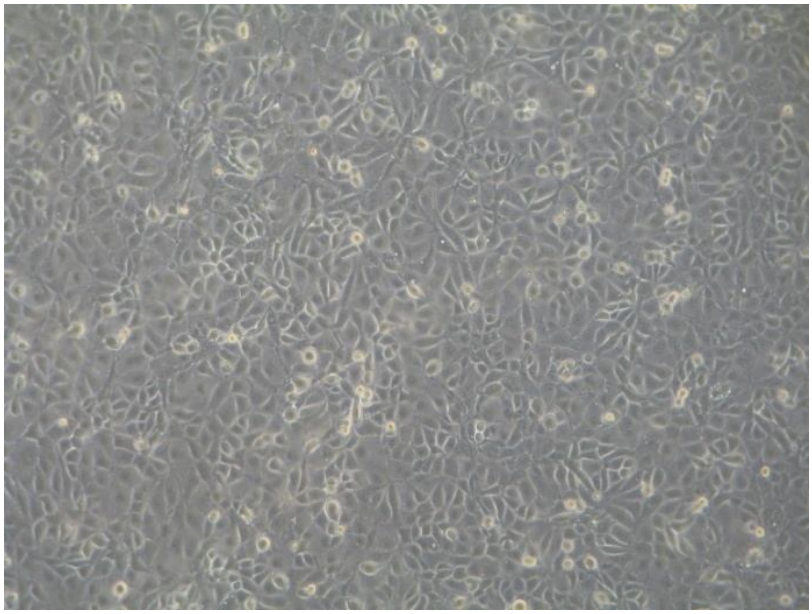


Figure 3.13 A) Morphology of HUVECs before 8 hour disturbed flow experiment downstream of the stagnation point 10X. **B)** Morphology of HUVECs after 8 hour disturbed flow experiment downstream of the stagnation point 10X. Regional cell lost (detachment) and cell elongation in the direction of the flow was observed.

A)



B)

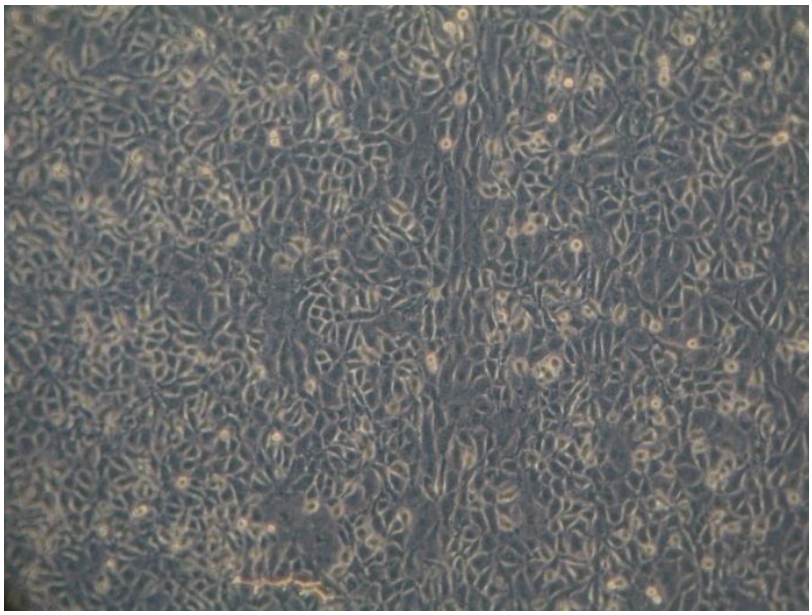
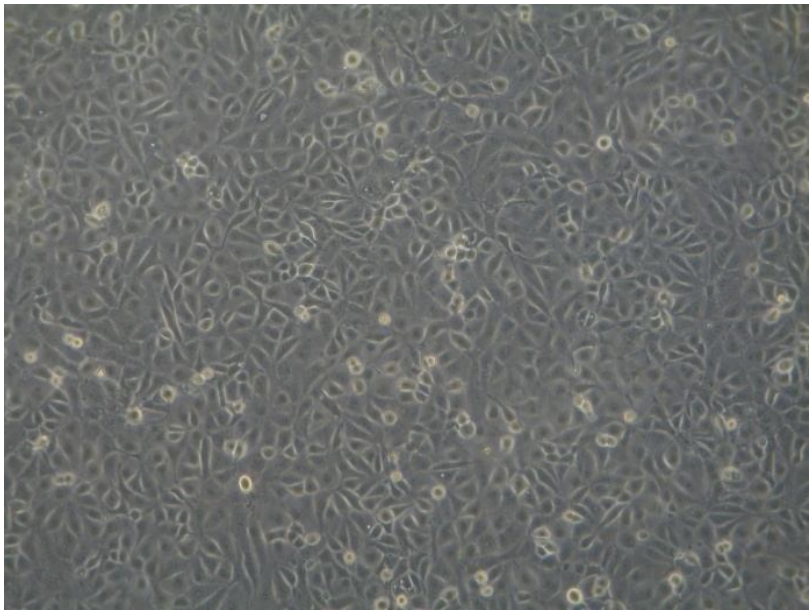


Figure 3.14 A) Morphology of HUVECs before 8 hour disturbed flow experiment middle region 10X. Cells have confluent monolayer. **B)** Morphology of HUVECs after 8 hour disturbed flow experiment middle region 10X. As shown in the figure, path like morphological change from polygonal to elipsodial along the direction of the flow was observed.

A)



B)

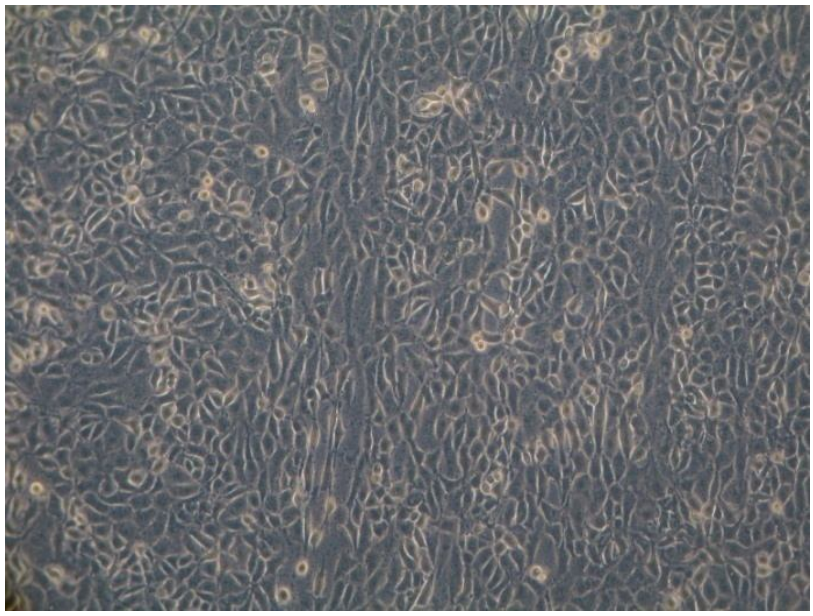


Figure 3.15 **A)** Morphology of HUVECs before 8 hour disturbed flow experiment down region 10X. **B)** Morphology of HUVECs after 8 hour disturbed flow experiment down region 10X. It is shown that cell elongation that observed in the middle region continued along the down region.

3.2.3 Q=150 ml/min 10 hour Disturbed Flow Experiment

The cells were subjected to 10 hour shear stress at flow rate 150 ml/min; we have 4.2 dynes/cm^2 shear stress. The same step flow model for disturbed flow experiment was used. Figures 3.16-A, 3. 17-A, 3.18-A and 3.19-A show the morphology of HUVECs before the experiment. After experiment the region that cells detached from the monolayer fairly closer to the step due to flow rate as shown figure 3.16-B. In figure 3.17-B downstream of the stagnation point, gaps between cells were observed.

A)

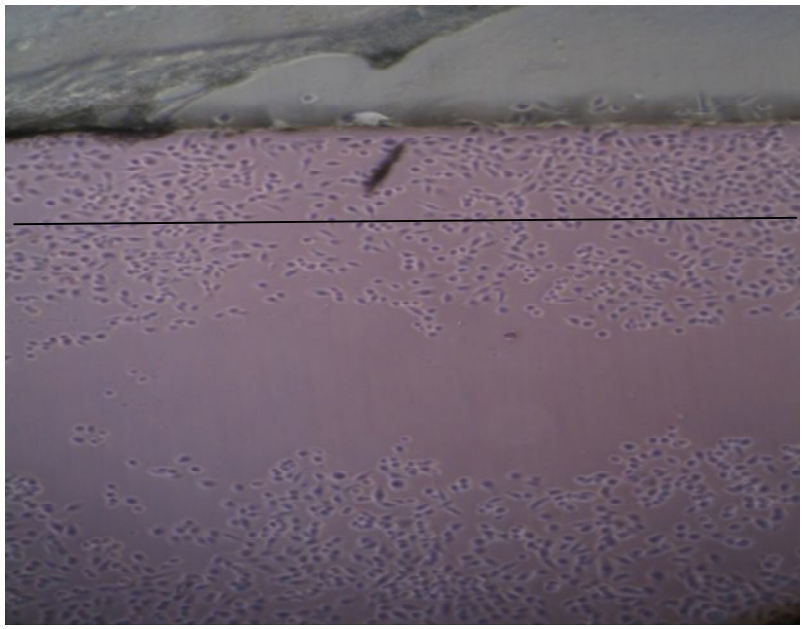


Figure 3.16 A) Morphology of HUVECs before 10 hour disturbed flow experiment upper region 5X. Dark line indicates the location of the step**. B)** Morphology of HUVECs after 10 hour disturbed flow experiment upper region 5X. In the stagnation point cell migration and detachment from the monolayer were observed. The region that flows fallen down resembles a long band across up-region of the coverslipe.

A)

B)

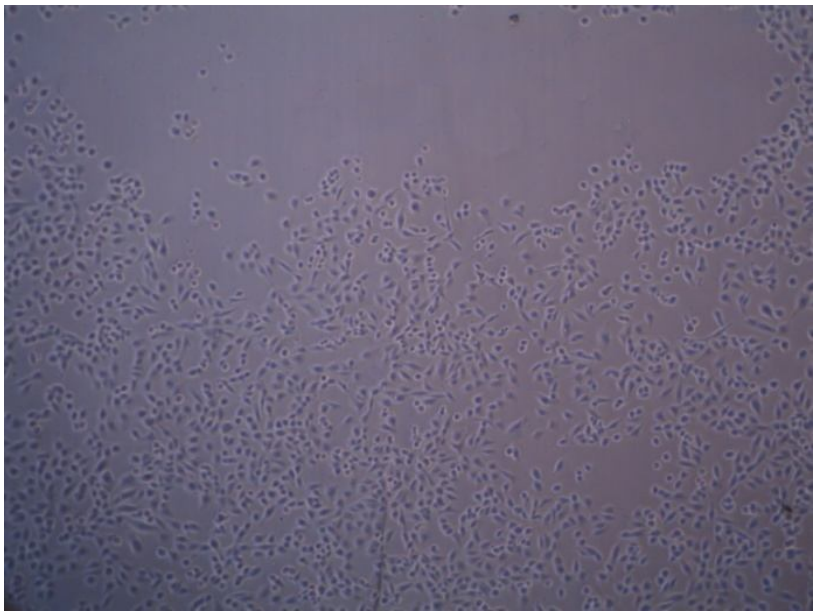
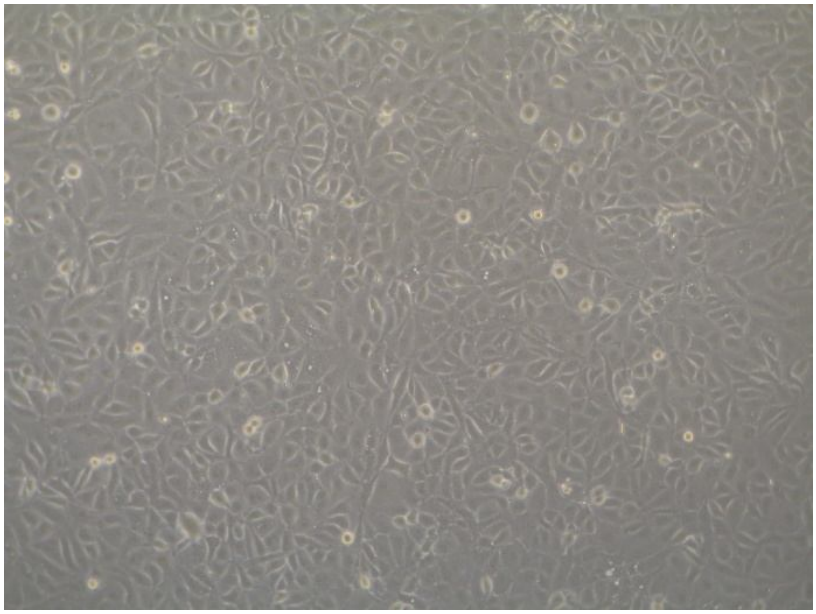


Figure 3.17 A) Morphology of HUVECs before 10 hour disturbed flow experiment on the downstream of stagnation point 5X. **B)** Morphology of HUVECs after 10 hour flow experiment on the downstream of stagnation point 5X. In this region gaps between cells were observed. Also, cell shape changed a bit from polygonal to elipsodial.

A)



B)

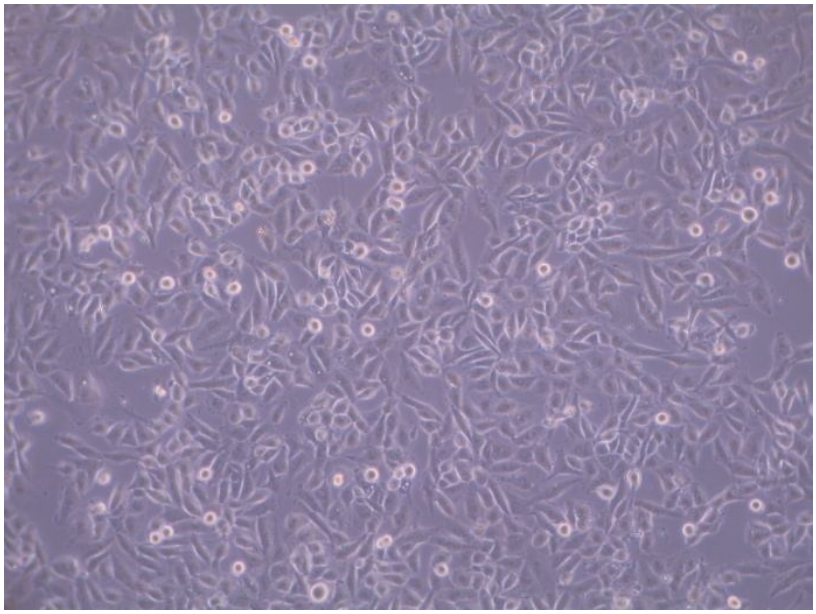
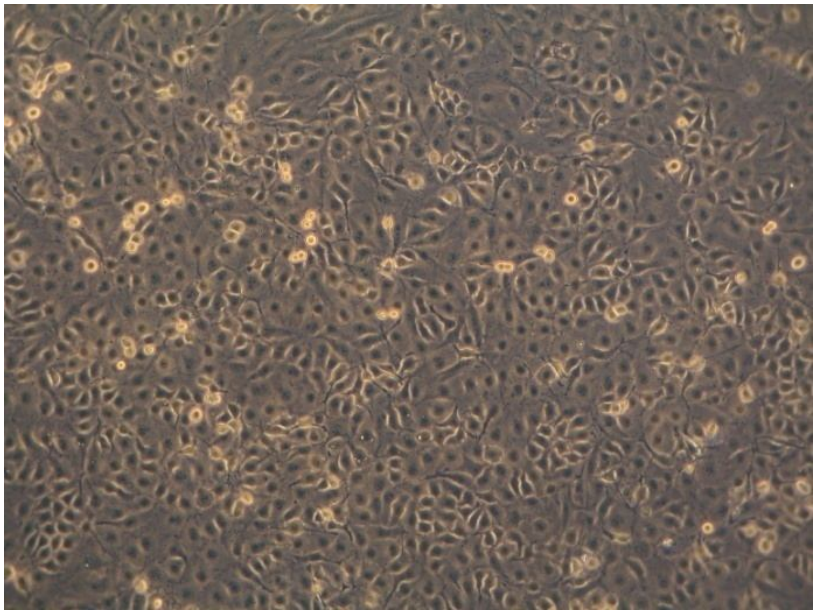


Figure 3.18 A) Morphology of HUVECs before 10 hour disturbed flow experiment middle region 10X. **B)** Morphology of HUVECs after 10 hour disturbed flow experiment middle region 10X. Cell shape changed into elipsodial but cell elongations are more intricate and not in the direction of flow.

A)



B)

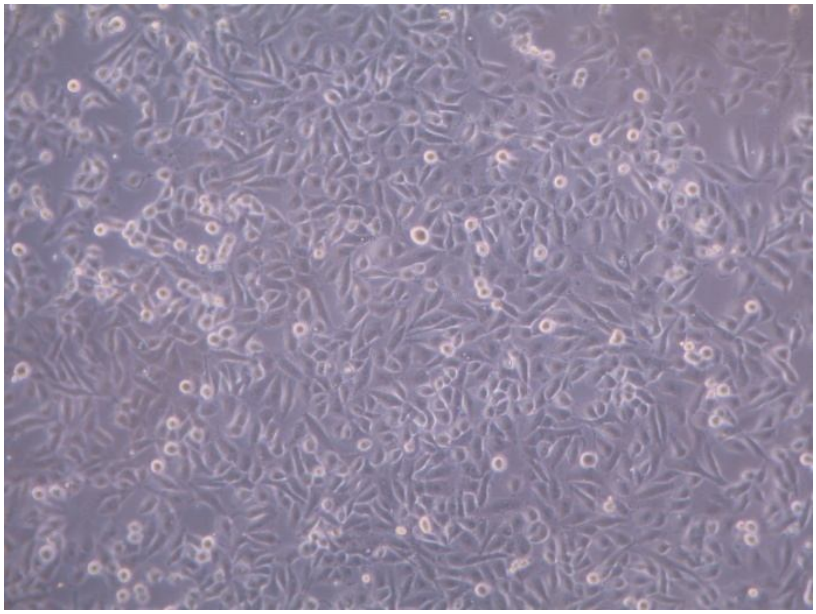


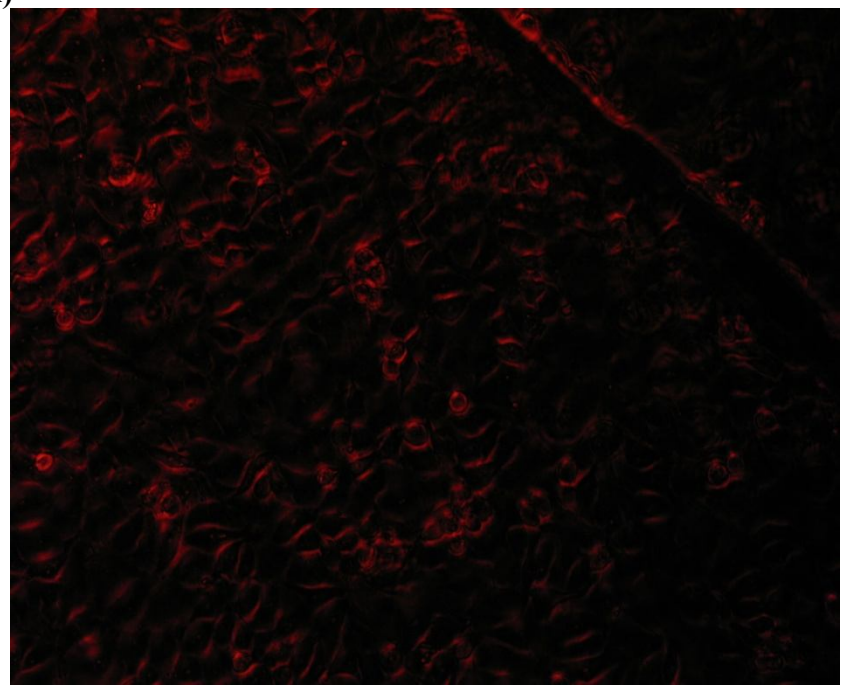
Figure 3.19 A) Morphology of HUVECs before 10 hour disturbed flow experiment down region 10X. **B)** Morphology of HUVECs after 10 hour disturbed flow experiment down region 10X. There is a remarkable change in HUVEC morphology, cells are elipsodial but they are so intricate.

3.2.4 Q = 100 ml / min 2 hour Disturbed Flow Experiment with VE-Cadherin

HUVECs were seeded and stained as mentioned protocol 2.2.6.1. Parallel – plate flow chamber and step model was used for the experiment. After immunofluorescense experiment, coverslipes settled on step model in the flow chamber. This monoclonal antibody binds to human VE-Cadherin. The antibody recognizes the extracellular domain of the protein. And with the 2nd Antibody GAM (Goat Anti Mouse)-IgG-Alexa (Mouse,red) we observed its red signal as shown figures 3.20 and 3.21.

Before experiment HUVECs are confluent and we can observe VE-Cadherin signals clearly on the cell membranes. Furthermore, step is seen in the figure 3.20-A. After flow experiment as shown figure 3.20-B, cells detached from the stagnation point and VE-Cadherin expression diminished in this region but figure 3.21-B shows a different result in this region VE-Cadherin expression increased and cell shape changed polygonal to elipsodial.

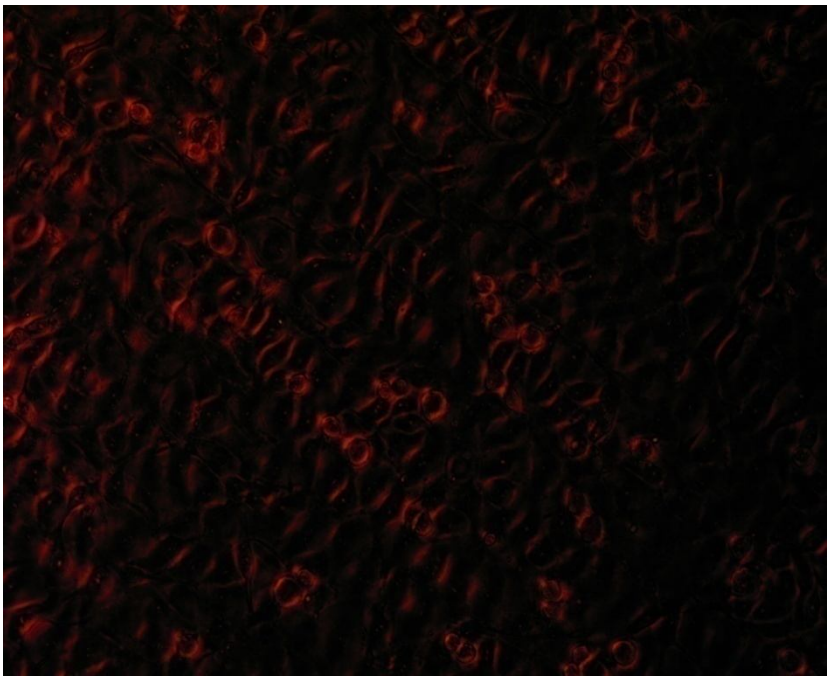
A)



B)

Figure 3.20 A) Morphology of HUVECs with VE-Cadherin staining before 2 hr flow experiment. **B)** Morphology of HUVECs with VE-Cadherin staining after 2 hr flow experiment. In the stagnation point, cells were detached from monolayer. Cell morphology became more polygonal and VE-Cadherin expression decreased.

A)



B)

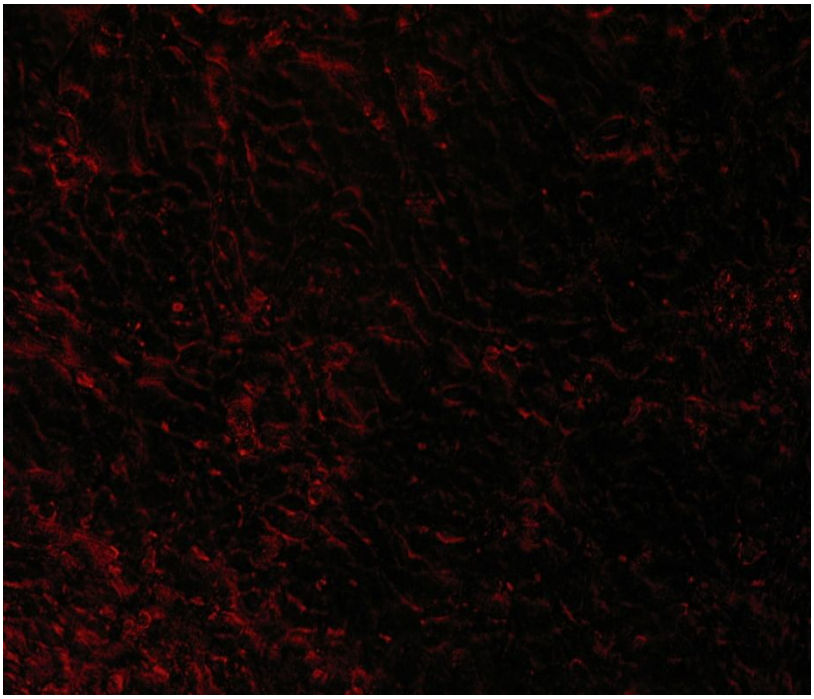
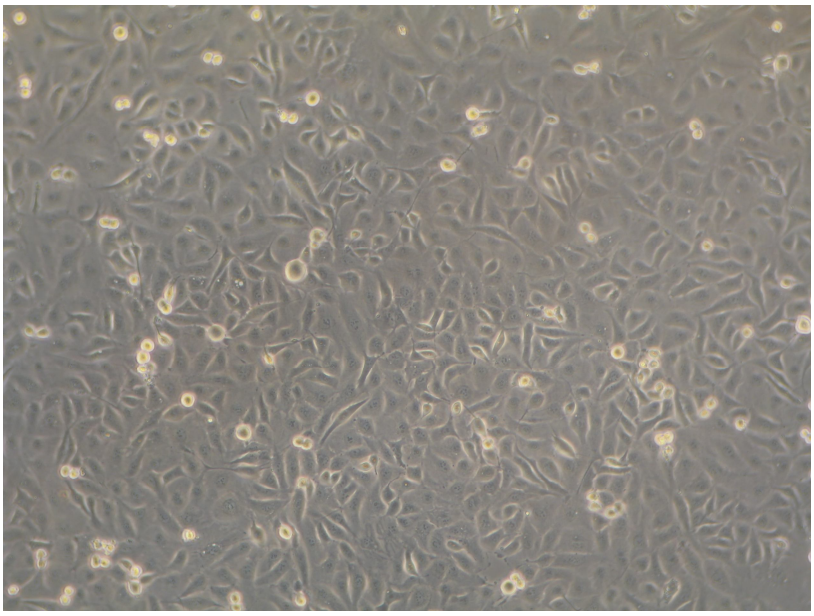


Figure 3.21 A) Morphology of HUVECs with VE-Cadherin staining before 2 hour flow experiment middle region 20X. VE-Cadherin staining at cell borders is quite visible. **B)** Morphology of HUVECs with VE-Cadherin staining after 2 hr flow experiment middle 20X. Cells aligned in the direction of flow and VE-Cadherin staining at cell borders is thinner. Reddish region in the figure show that cells may diffuse antibody into the cell by the influence of flow.

3.2.5 Q=500 ml/min 24 hour Steady Flow Experiments with Heparinase III degraded HUVECs

Heparan sulfate GAGs were removed with Heparinase III enzyme as mentioned protocol 2.2.7.1. After the enzyme treatment, coverslipes were placed on the parallel-plate flow chamber and then HUVECs were subjected to 24 hour shear stress at flow rate 500 ml/min. Figures 3.22A and 3.23A shows the morphology of HUVECs before the experiment. Figures 3.22B and 3.23B shows the heparinase degraded HUVECs after the flow experiment. Heparinase degraded HUVECs did not align in the flow direction and they continued their proliferation as if there were no flow present.

A)



B)

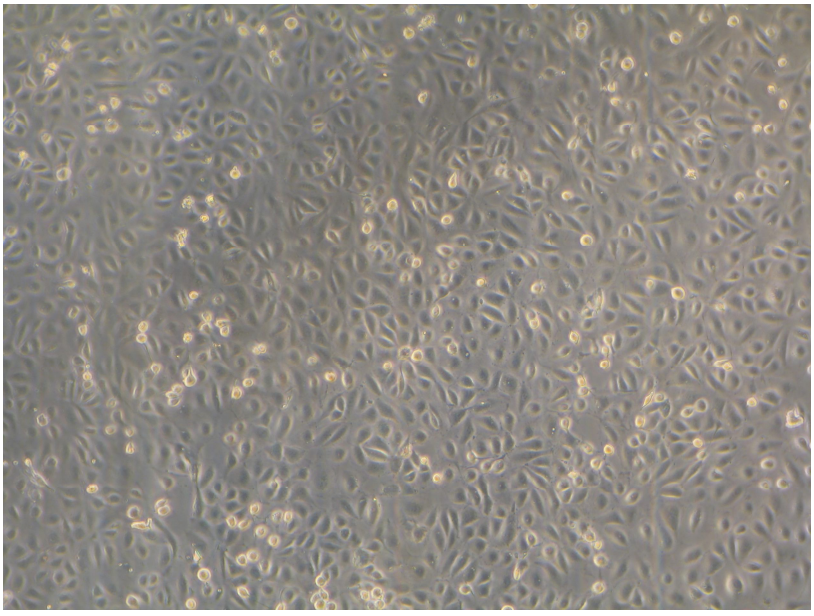
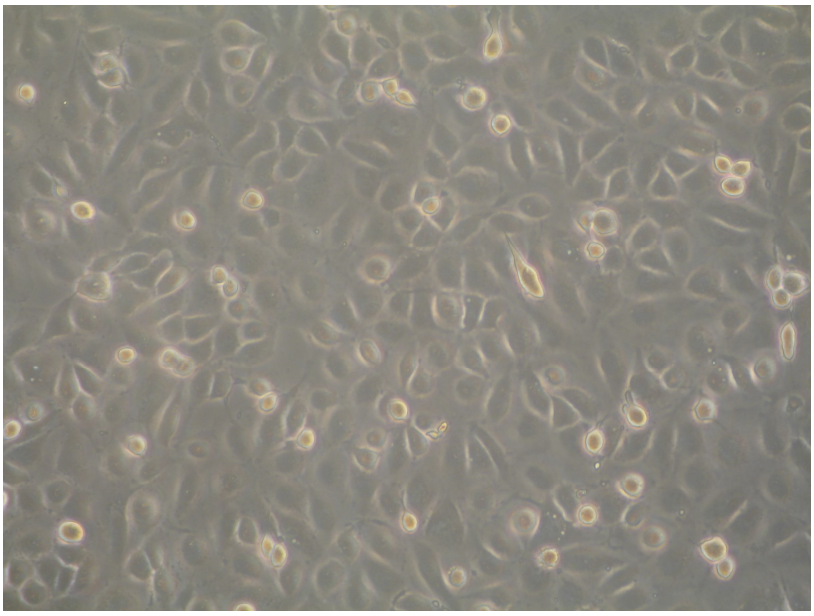


Figure 3.22 A) Morphology of HUVECs before enzyme treatment and 24 hour flow experiment up region 10X. **B)** Morphology of HUVECs after enzyme treatment and 24 hour flow experiment up region 10X. Heparinase treated cells did not align in the flow direction and HUVEC morphology did not change with the effect of flow.

A)



B)

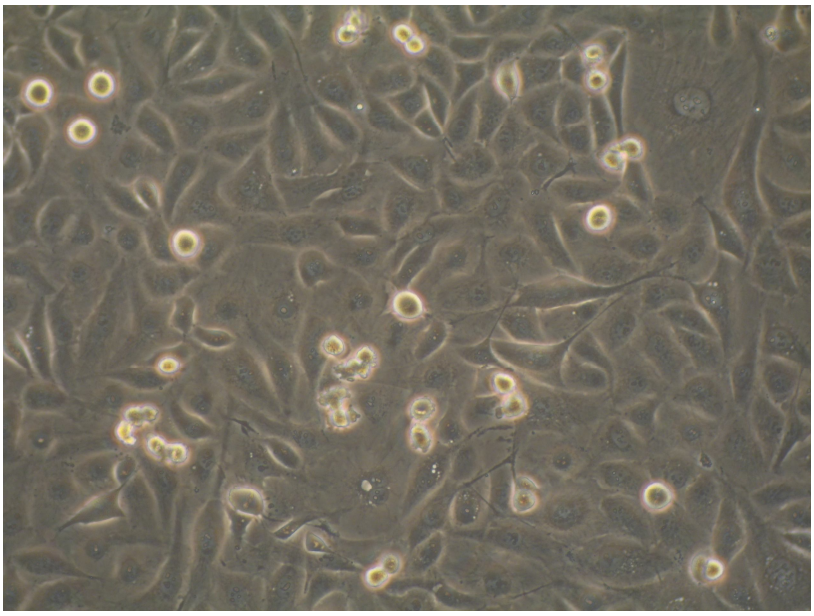
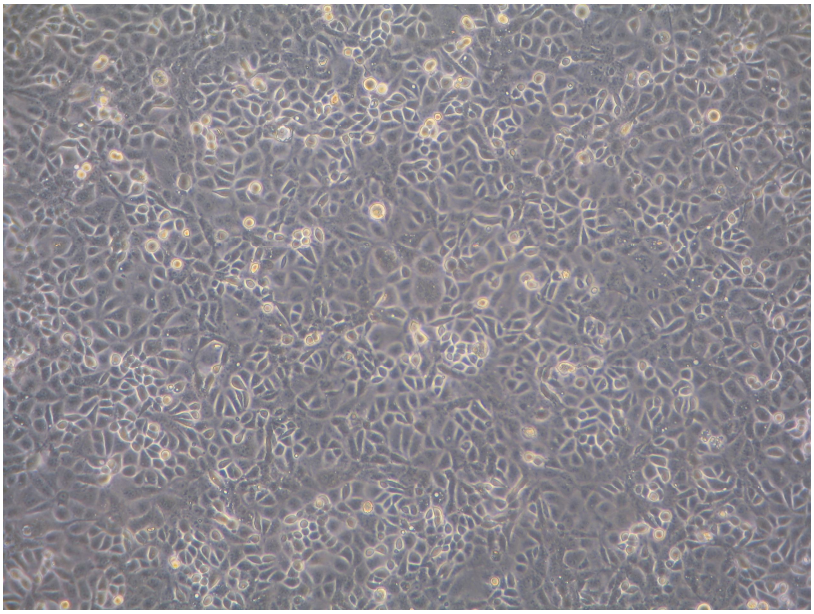


Figure 3.23 A) Morphology of HUVECs before enzyme treatment and 24 hour flow experiment middle region 20X. **B)** Morphology of HUVECs after enzyme treatment and 24 hour flow experiment middle region 20X. HUVECs were proliferated as if there were no flow present. Cells did not align and elongate in the flow direction. Also, little particles were observed on the cell monolayer.

3.2.6 Q=500 ml/min 24 hour Steady Flow Experiment with Chondrotinase Degraded HUVECs

Condrotin sulfate GAGs were removed with Chondrotinase ABC enzyme as mentioned protocol 2.2.7.2. Condrotin degraded HUVECs were exposed to 24 hour steady flow rate at 500 ml/min. Figures 3.24A, 3.25A and 3.26A show the HUVEC monolayers in the static condition before the experiment and enzyme treatment. Figures 3.24B, 3.25B and 3.26B show the HUVEC morphology after the enzyme treatment and flow experiment. In these figures cell shape did not change with the flow direction. Also, particles were observed on the cell monolayers. Figures 3.27A and 3.27B shows the degraded particles on the monolayer when the microscope light was switched off. These particles were blue color.

A)



B)

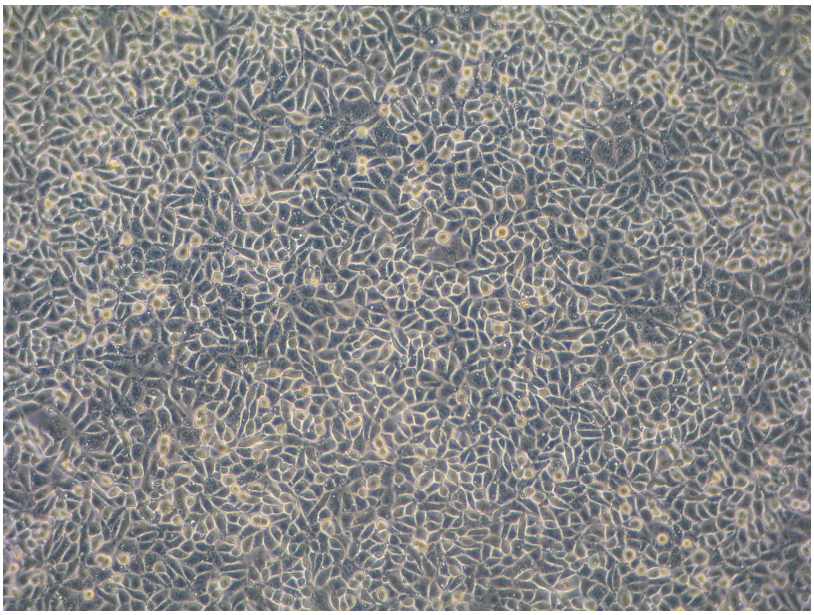
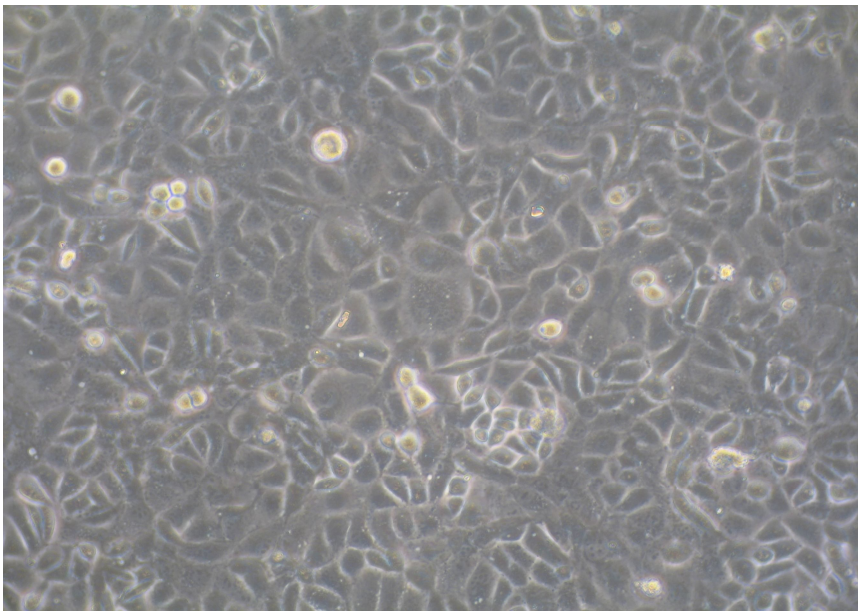


Figure 3.24 A) Morphology of HUVECs before enzyme treatment and 24 hour flow experiment up region 10X. HUVECs have a confluent monolayer. **B)** Morphology of HUVECs after enzyme treatment and 24 hour flow experiment upper region 10X. Cell shape did not change and they were not influenced from the flow effect. Particles were observed on the cell monolayer.

A)



B)

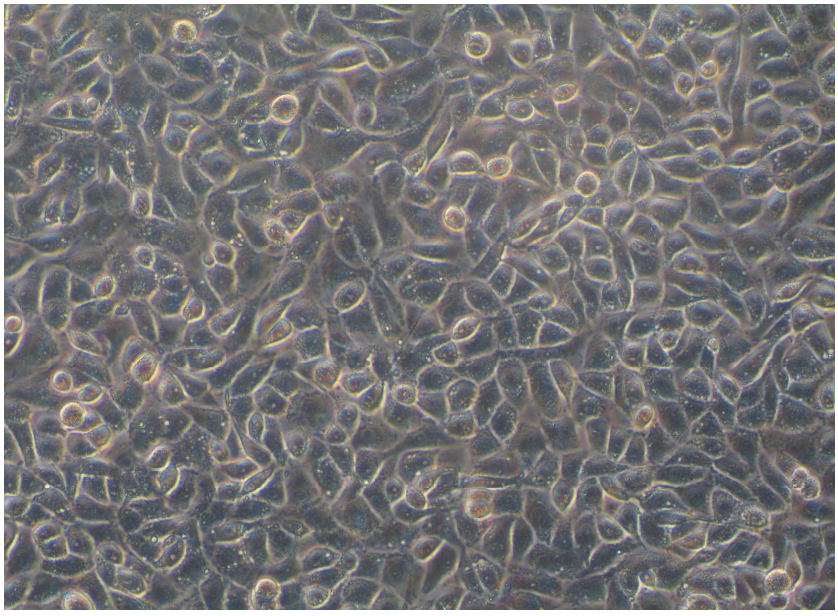
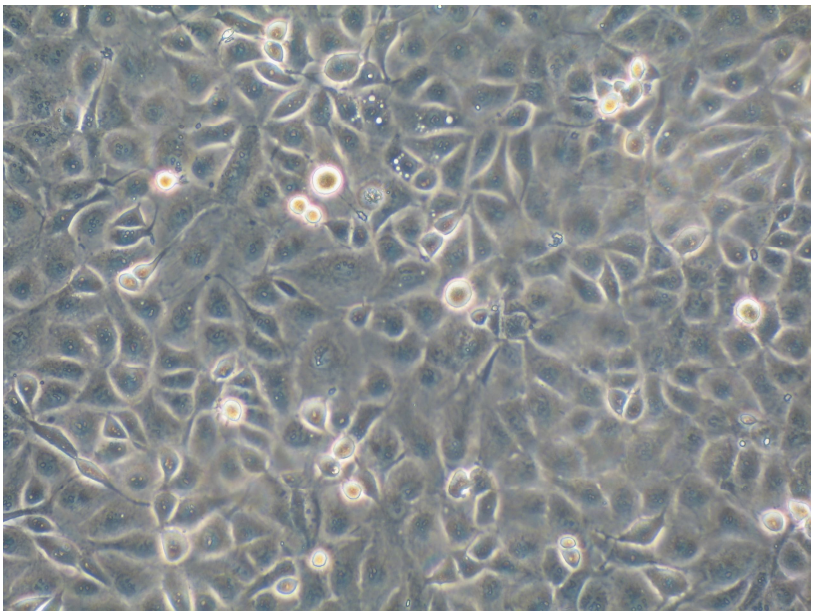


Figure 3.25 A) Morphology of HUVECs before enzyme treatment and 24 hour flow experiment middle region 20X. **B)** Morphology of HUVECs before enzyme treatment and 24 hour flow experiment middle region 20X. Cells did not align in the flow direction and little particles inside the cells were observed clearly.

A)



B)

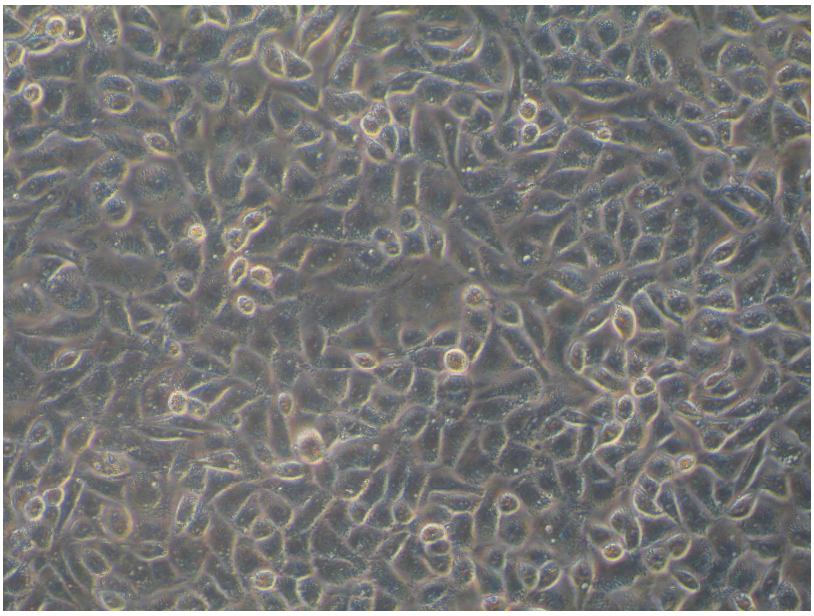


Figure 3.26 A) Morphology of HUVECs before enzyme treatment and 24 hour flow experiment down region 20X. **B)** Morphology of HUVECs after enzyme treatment and 24 hour flow experiment down region 20X. Cell shape change was not observed. Particles on the cell monolayer were shown.

A)

B)

Figure 3.27 A) Degraded particles on the HUVECs monolayer after Chondrotinase treatment and 24 hour flow experiment 10X. **B)** Degraded particles on the HUVECs monolayer after Chondrotinase treatment and 24 hour flow experiment 20X.

CHAPTER 4

DISCUSSION

Flow experiments inside the flow chambers have been started approximately twenty five years ago to understand the effects of hemodynamic forces on cell structure and function. There is considerable evidence to suggest that hemodynamics play an important role in the pathogenesis of vascular disease such as atherosclerosis. Studying hemodynamic parameters in arterial system in vivo is very hard so that researchers started to get interested in-vitro models to study the effects of arterial hemodynamic forces on the vascular wall cells. In 1981, an *in vitro* system called cone-plate flow system was developed by Dewey and his colleagues to study shear stress effects on endothelial cells. Recent years another flow system apparatus called parallel-plate flow chamber is used for *in vitro* flow experiments. During experiments, we used parallel–plate flow chamber that originally designed in our laboratory

In vivo, ECs are constantly exposed to the fluid shear stress generated by blood flow. Disrupted flows occur at the curvatures and branch points of blood vessels. These regions tend to develop atherosclerotic regions. *In vitro* and *in vivo* studies have shown contrasting effects between laminar shear flow with a definite direction (seen in the straight part of the arterial tree) and disturbed shear flow without a clear direction (seen at arterial branch points and curved regions) in terms of their signal transduction, gene expression, structure, and functions

We studied with HUVECs by exposing their monolayers to altered shear stress and tried to find their responses to steady laminar flow and disturbed flow. The effects of fluid shear stress on EC morphology have been determined by many researchers (Dewey et al., 1981, Levesque MJ and Nerem RM, 1985, C.G.Galbraith et al., 1998, PF Davies et al., 1995). They found that ECs aligned and elongated in the direction of fluid flow. We have observed the similar results at the flow rates of 1000 ml/min and 1500 ml/min. In contrast, in disturbed flow regions, cells became more polygonal. We did disturbed flow experiments generating a step flow by sticking two coverslips to each other that the coverslip placed at the bottom has a

longer size than the upper one. One of the coverslips thickness was 1 mm and the other one was 0.1 mm. HUVECs were seeded on the unsticked region of the bottom coverslip. Then, the flow was allowed to flow over the cells. Our disturbed flow experiments demonstrated that HUVECs detached from monolayer in the stagnation point where flow fallen down generating a band lying down throughout the stagnation point. Downstream of stagnation point region cells elongate in the direction of flow. This could be as a result of the movement of the adhesion proteins such as VE-Cadherin proteins on the cell membrane along the direction of the fluid flow.

Endothelial cells respond to shear stress not only with their cytoskeleton but also their adherens junctions. Our immunofluorescense data for VE-Cadherin showed a decrease at VE-Cadherin signaling after HUVECs were exposed to fluid shear stress in the stagnation point at the flow rate of 100 ml/min. Moreover decrease in the VE-Cadherin staining on the stagnation point caused the cells to detach from monolayer as an influence of the flow. Furthermore, cell elongation occurred at the middle region of the coverslip. VE-Cadherin signal at the elongated cell borders was less dense than the regular cell borders. As a result of these experiments show that VE-Cadherin can be considered as the mechanosensor inside the arterial tree of human cardiovascular system. Our experimental results are similar results compared to the results of laminar flow experiments in the literature however different findings for disturbed flow.

We examined the glycocalyx function in endothelial cell shear stress responses by using Heparinase III to cleave heparan sulfate GAGs and Chondrotinase ABC to cleave Condrotin sulfate GAGs on the cell surface. Our previous steady flow experiment datas showed that ECs align in the direction of the flow after subjected to flow rates 1000 ml/min and 1500 ml/min at 24 hour. But Heparinase and Chondrotinase degraded cells did not align in the direction of the flow.

CHAPTER 5

CONCLUSION

In this thesis, we started to investigate shear stress effects on endothelial cells and glycocalyx. We originally designed a parallel plate flow chamber in our laboratory. Our flow experiments are done using this chamber.

HUVECs were exposed to steady laminar flow at flow rates 1000 ml/min and 1500 ml/min for 24 hour. After 1000 ml/min steady flow experiment cells elongated and aligned in the flow direction. After 1500 ml/min steady flow experiment, gaps between cells and regional cell elongation were observed.

We examined disturbed flow effects on HUVECs generating a step model that is formed by sticking two coverslips each other. HUVECs were exposed to step flow at flow rates of 250 ml/min 5 hour, 500 ml/min 8 hour, and 150 ml/min 10 hr. After 250 ml/min 5 hour flow experiment, cells are dragged by the flow in the stagnation point or reattachment sites. In middle and down regions cells became more polygonal. After 500 ml/min 8 hour flow experiment, we observed cell dead in the downstream of stagnation point. This result is not seen by former researchers. It shows that cell exposed to high flow rates is died. Also, cells are aligned in flow direction in middle and down regions of the coverslips. After 150 ml/min 10 hour flow experiment, cells detached from monolayer in stagnation point but stagnation point was closer to step due to low flow rate. Cell shape changed polygonal to ellipsoidal in middle and down regions of the coverslips but cell elongation was not in the flow direction.

Immunofluorescense experiments were optimized. After 100 ml/min 2 hour step flow experiment with VE-Cadherin staining, we observed that In stagnation point cells detached from monolayer. Cell shape became more polygonal and VE-Cadherin expression decreased. In contrast, in middle region cell shape changed elipsodial and VE-Cadherin expression

increased. Cell membrane structure was damaged by the flow that so antibody diffused in the cell.

This study is the first experimental study to understand the effects of flow shear stresses on endothelial cells under disturbed flow conditions in Turkey. The study will continue to better understanding the adhesion protein (VE-cadherin) behavior under different ranges of shear stresses. Additionally computational simulation models will be studied at different flow rates.

Heparinase III and Chondrotinase ABC degraded HUVECs were exposed to steady flow at flow rate 500 ml/min for 24 hour. Heparinase and Chondrotinase treatment blocked the cell alignment in response to flow. Cell shape did not change to elipsodial. As a result of these experiments glycocalyx is a mechanotransducer and it is necessary for responding shear stress. Degragation of glycocalyx reduced shear stress effects on the cell surface. Our results suggest that HS and CS GAGs have an important role in mechanotransduction.

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