Glycocalyx acting as a mechanotransducer of fluid shear stress

by

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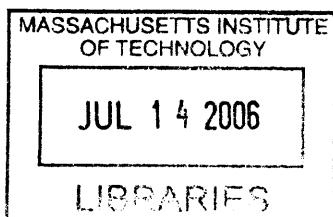
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Abstract

It is widely recognized that fluid shearing forces acting on endothelial cells (ECs) have a profound effect on EC morphology, structure and function. Recent investigations in vivo have indicated the presence of a thick endothelial surface layer, also called the glycocalyx, with an estimated thickness on the scale of 0.5 μm, which restricts the flow of plasma and can exclude red blood cells and some macromolecular solutes.

Based on our current understanding of signal transduction, we expect that the glycocalyx plays an essential role as a possible mechanotransducer of fluid shear stress to the actin cytoskeleton of the endothelial cells. We examined whether removing the glycocalyx can affect shear-induced cellular response, e.g. cell migration speed and wound edge healing. More specifically, we compared the two major components of the glycosaminoglycans in the glycocalyx, which are heparan sulfate GAGs and chondroitin sulafte GAGs.

Our results showed that the removal of heparan sulfate GAGs has a much greater impact on the shear induced response compared to chondroitin sulfate GAGs, and the short term crawling speed response is highly suppressed in the first case. Therefore, it is highly possible that heparan sulfate GAGs is involved in the shear-induced signaling pathway.

Thesis Supervisor: C. Forbes. Dewey, Jr.

Title: Professor of Mechanical Engineering

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List of Abbreviations

ABPs	actin binding proteins
BAEC	bovine aortic endothelial cells
CSGAGs	chondroitin sulfate glycosaminoglycans
cABC	chondroitinase ABC
FAs	focal adhesions
GAGs	glycosaminoglycans
FRAP	fluorescent recovery after photobleaching
HUVEC	human umbilical endothelial cells
HSGAGs	heparan sulfate glycosaminoglycans
HepI	heparinase I
HepIII	heparinase III
PAF	photoactivation of fluorescence

Chapter 1. Introduction

1. Endothelium

The luminal surface of human arteries is lined with a continuous layer of vascular endothelium (Figure1). The endothelial cells are always subject to fluid flow, and dynamically exchanging molecules with plasma. In arteries, shear stress has been demonstrated to regulate both acute vasoregulation and chronic adaptive vessel remodeling and is strongly implicated in the localization of the atherosclerotic lesions. Therefore, endothelial biomechanics and shear-induced mechanotransduction is of great importance in vascular physiology and pathology.
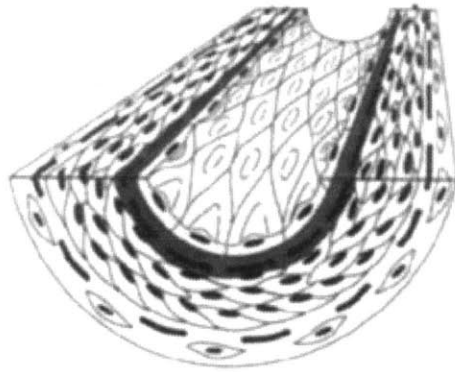

Fig1. Vessel structure. An endothelial cell monolayer lines the lumen of the vessel, and is separated from smooth muscle cells by a layer of connective tissue. (Ross 1995)

1.1.1 Endothelial cells

The endothelial cells play various functional roles, and may participate in atherogenesis when disturbed (Ross 1995). These functions include:

1. provision of a nonadherent surface for leukocytes and platelets;
2. formation and secretion of various molecules and cytokines;
3. formation and maintenance of tissue matrix, including collagen, elastic fibers and proteoglycans that the basal membrane lines upon;
4. control of the exchange of nutrients and fluid between plasma and the artery wall;
5. maintenance of vascular tone by releasing small vasodilatory molecules such as nitric oxide (NO) and prostacyclin (PGI_2), and vasoconstrictive molecules such as endothelin (ET) and angiotensin-II (A-II);
6. provision of anticoagulant and procoagulant activities.

Dysfunction of the endothelial cells may lead to the development of atherosclerosis in early stage.

1.1.2 Shear stress effects on endothelial cells

Endothelial cells are continuously exposed to fluid forces. These forces can be analyzed in two components:

1) pressure, which is acting normal to the luminal surface and transmitted to the elastic structures lying under endothelium;
2) shear stress, which is acting tangential to the luminal surface and absorbed by the endothelial monolayer.

Spatial and temporal variations in the hemodynamic shear stresses create a complex force environment between the luminal surface of the cells and plasma. Investigations in the past decades have shown various morphological and functional responses of endothelial cells to either laminar or turbulent shear stresses. Shear stress is known to have a profound impact on endothelial cell

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morphology, cytoskeleton organization, membrane mechanical properties, intracellular signaling, endocytosis, cell cycle entry, activation of ion channels, messenger RNA, protein synthesis and mobilization of intracellular calcium. According to a number of reviews (Davies 1995; Ishida, Takahashi et al. 1997; Schnittler 1998; Takahashi, Shimada et al. 1998; Gimbrone, Topper et al. 2000; Barakat and Lieu 2003; Cunningham and Gotlieb 2005) of shear stress effects on endothelial cells. The major responses will be summarized below.

1. Rapid flow-induced responses.

Within the first minutes of flow application, intracellular free calcium concentration has a sharp increase (Shen, Luscinskas et al. 1992). In the same time frame, whole cell height and nuclear height are reduced by about 1μm (Stamatas and McIntire 2001). Whole cell height changes may facilitate reduction of shear stress gradients on the luminal surface, whereas nuclear structural changes may be important for modulating endothelial growth rate and metabolism. The endothelial monolayer surface is smoothed in the first hour (Seebach, Dieterich et al. 2000; Schnittler, Schneider et al. 2001), and microvilli present on the apical endothelial surface under static conditions disappear, leaving a smooth, glassy contour(Satcher, Dewey et al. 1997). The motility of the EC slows down by 50%, while the actin filament turnover rate increases and the amount of polymerized actin decreases, accelerating actin filament remodeling in individual cells composing a confluent endothelial monolayer (unpublished results by Osborn and others 2005).

2. Long term flow-induced responses.

For hours after the flow, focal adhesion rearranges (Thi, Tarbell et al. 2004),and cell-cell junctions redistributes, e.g. Connexin 43 increased and redistributed from junctions to cytoplasm (DePaola, Davies et al. 1999). Cytoskeleton remodeling occurs in response to shear. Basally-located dense peripheral bundles of F-actin stress fibers dissolve shortly after shear stress exposure, and reform hours later under the apical membrane, aligned with the long axis of the

cells (Satcher, Bussolari et al. 1992; Barbee, Mundel et al. 1995). These structural changes reduce the peak shear stesses imposed on indivual cells , and cause elongated endothelial cells to become more resistant to micropipette surface deformations (Sato, Ohshima et al. 1996). After 24 hours of flow application, the monolayer of EC aligned in the direction of flow (Dewey 1984).

3. Laminar flow v.s. disturbed flow

Hemodynamic forces imposed by the flow of blood play an important role in the physiology and pathology of the vascular wall. Studies have shown that the vessels most affected with atherosclerosis are endowed with particular geometric architectures and share unusual blood flow characteristics (Nerem 1992). Laminar shear stress is atheroprotective for endothelial cells, whereas nonlaminar, disturbed or oscillatory shear stress correlates with develop of atherosclerosis and neointimal hyperplasia (Nerem 1992). ECs responses to laminar flow and disturbed flow differ. Early study (Davies, Remuzzi et al. 1986) has shown that laminar shear stresses induced cell alignment in the direction of flow without initiating the cell cycle. In contrast, turbulent shear stresses stimulated substantial endothelial DNA synthesis in the absence of cell alignment, discernible cell retraction, or cell loss. Other investigations have determined that oscillatory shear alters endothelial hydraulic conductivity and nitric oxide levels (Hillsley and Tarbell 2002). Calcium response of endothelial cell monolayers subjected to pulsatile and steady laminar flow also differs (Helmlinger, Berk et al. 1996) in the way that laminar flow increased intracellular free calcium concentration while pulsatile flow failed to increase calcium concentration.

It is still unclear how endothelial cells sense the flow. There are a few studies of shear stress mechanosensors that have been conducted so far (Fisher, Chien et al. 2001), including membrane receptor kinase, integrins, G proteins, ion channels, intercellular junction proteins, membrane lipids and the cytoskeleton (Wang, Butler et al. 1993; Malek and Izumo 1996; Chen, Li et al. 1999; Chien 2003; Mazzag, Tamaresis et al. 2003). Recent investigations (Florian, Kosky et al.

2003; Mochizuki, Vink et al. 2003; Thi, Tarbell et al. 2004) also suggest that glygcocalyx could also serve as a mechanosensor to fluid shear stress.

1.1.3 Actin dynamics in endothelial cells

When subjected to fluid shear, cells composing an endothelial monolayer change from polygonal shapes into torpedo shapes (Dewey, Bussolari et al. 1981) and remodel their cytoskeleton that is mainly composed of F-actin filaments(Satcher and Dewey 1996). F-actin is a polymer of actin monomers and actin-biding proteins (ABPs), and it polymerizes and depolymerizes in an ATP-powered cycle (Figure 2).

Actin is the most abundant cytoplasmic protein, and is the main component of cell cytoskeleton. Filament polymerization grow preferentially at "barbed" end, while disassemble at "pointed" end. ABPs regulate actin filaments by sequestering, severing, capping and nucleation.
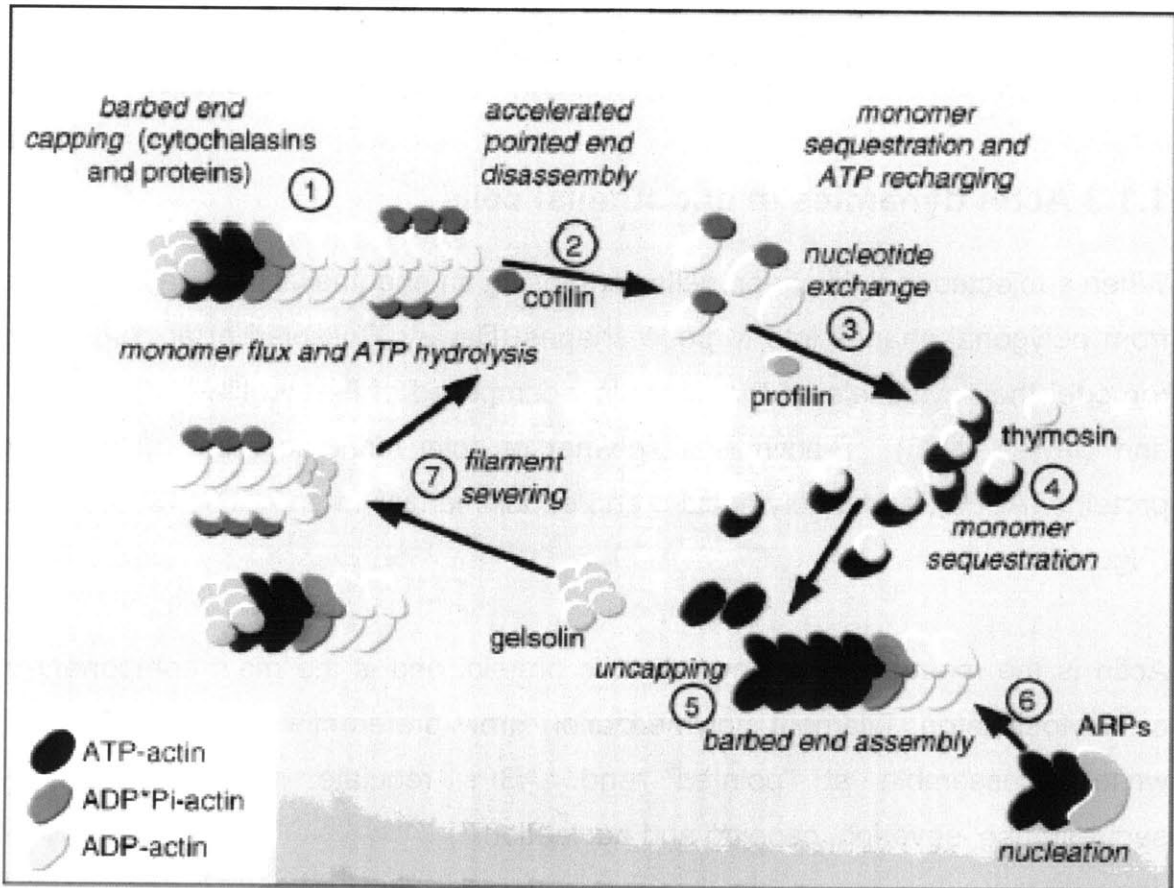
Fig2. Actin binding proteins controlling actin dynamics by capturing and severing filaments. (McGrath, Osborn et al. 2000)

As cells crawl, actin polymerizes near the plasma membrane of expanding peripheral cytoplasm and depolymerizes elsewhere. Thus, cell mobility can be evaluated by important parameters such as actin filament lifetime, the diffusivity of actin monomer and the fraction of actin monomers compared to actin polymers. Using the techniques of photoactivated fluorescence (PAF) or fluorescence recovery after photobleaching (FRAP) of microinjected actin derivatives (McGrath et al 1998), actin turn over rates can be measured as an indicator of the extent of cytoskeleton remodeling. The characteristic time for filament turnover is about 6 minutes for subconfluent endothelial cells. Resent investigation (unpublished results from Osborn) has also shown an increase of actin turn over rates and decrease of the amout of polymerized actin filaments, which accelerates the actin filament remodeling in individual cells composing a confluent endothelial

monolayer. Remodeling of actin is an important mechanism for the cell to distribute the stress by minimizing the load and optimizing its structure to resist the surface mechanical load, and in vivo observations have found endothelial cell shape and arterial atherogenicity may be markedly different between cells located just a few cell diameters away from each other because of their surface topography (Dewey 2002; Feldman, Ilegbusi et al. 2002; Davies, Zilberberg et al. 2003). Therefore, changes in the actin network structure and associated remodeling process may have an important impact on cellular mechanotransduction and atherogenic process.

1.2 Glycocalyx

The glycocalyx is a layer composed of various transmembrane macromolecules locating on the apical surface of endothelial cells. Since the glycocalyx was observed *in vivo* (Luft 1966; Dall, Barnes et al. 1987; Clough, Michel et al. 1988; Clough 1991; Vink and Duling 1996), people have started to investigate its important physiological functions. The glycocalyx has been found to be critically involved in many processes such as regulation of blood flow, inflammatory responses and blood coagulation (Pries, Secomb et al. 2000). We are especially interested in how fluid shear stress is transmitted through the glycocalyx to the cytoskeleton since the externally-imposed shear force must be continuously transmitted from the apical surface beginning with the glycocalyx to the basal attachments of the cell to its substrate.

1.2.1 Observation of the glycocalyx

The glycocalyx was first observed by electron microscopy more than 50 years ago (Chambers and Zweifach 1947). At that time, it was characterized as a thin layer of molecules of about 20 nm thick, mainly composed of membrane-bound proteoglycans and glycoproteins. However, since the methods for the quantitative intravital study of microvascular blood flow using light microscopy have been well developed, surprising discrepancies have been observed between haemodynamic parameters and the values expected on the basis of prevailing biophysical concepts (Pries, Secomb et al. 1990). Subsequent investigations using staining techniques in electron microscopy also showed a much thicker endothelial surface layer (Luft 1966; Clough, Michel et al. 1988)(Figure 3). Because the fragile structure of the glycocalyx with high water content may collapse during the preparing procedure of electron microscopy, the thickness of the glycoclayx may be still well under-estimated.

In 1996, Vink and Duling (Vink and Duling 1996) used FITC-dextran to label the free-flowing plasma and for the first time in vivo, they provided the visual

evidence for the existence of a continuous glycocalyx with a thickness of about 0.4-0.5 um (Figure 4). Since then, a number of researchers have put great enthusiasm into the research of the glycocalyx.
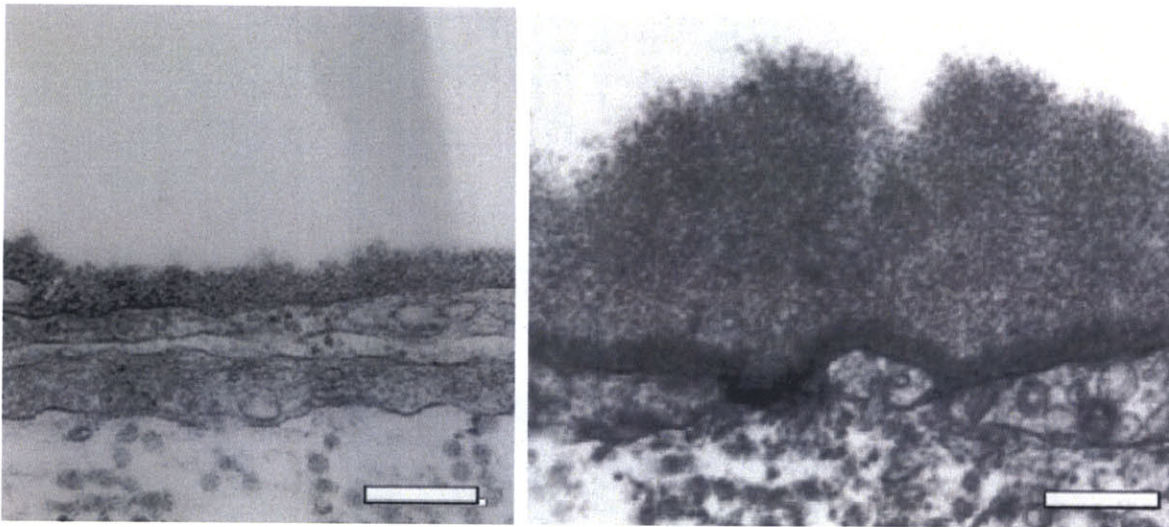
Fig 3. Overview micrographs of conventionally prepared plastic-embedded capillaries from the frog mesentery (Clough and Michel 1988).
Left: this is a "normal vessel"
Right: this vessel has been subjected to mild thermal injury and has become inflamed.
Both micrographs show the lumen (top), glycocalyx (centrally placed), endothelial cells (below the glycocalyx), and the collagenous extracellular matrix (lower area). Scale bars are 180 nm in both images.
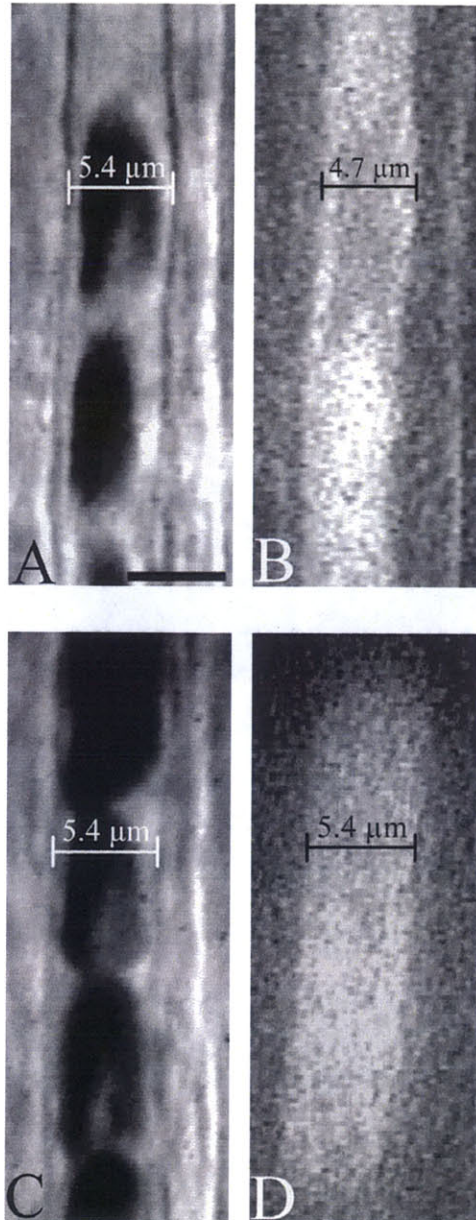
Fig 4. Digitized images of a capillary segment before (A and B) and after (C and D) continuous exposure to epi-illumination for 5 minutes. Before epifluorescent treatment, RBC width (A) and the width of the FITC-dextran column (B) were significantly smaller than the anatomic capillary diameter (A). Treatment of the capillary with epi-illumination increased the width of RBCs (C) and the FITC-dextran column (D), without a significant effect on the anatomic capillary diameter (C). The scale bar in the lower right corner of panel A represents 5 µm.

Because of tissue movement, the lower two images are shifted upward by 5 μm relative to the upper two images. A field diaphragm in the epi-illumination pathway was used to minimize the tissue area that was exposed to continuous epi-illumination, preventing excitation of fluorochromes in the upper and lower capillary ends in panel D. (Vink and Duling 1996)

1.2.2 Composition of the glycocalyx

The endothelial surface is covered by a large variety of extracellular domains of membrane-bound molecules, which together constitute the glycocalyx. These include proteins, glycolipids, glycoproteins and proteoglycans, many with exposed charged groups. Examples are cell adhesion molecules, e.g. selectins and integrins, involved in immune reactions and inflammatory processes. The composition and the physical properties of glycocalyx, including effective thickness, are influenced by adsorption of plasma proteins. The hypothetical composition of glycocalyx proposed by A.R.Pries (Pries, Secomb et al. 2000) is shown in Figure 5. A comparatively thin (50-100 nm) region on the endothelial surface is dominated by molecules (glycoproteins and proteoglycans) bound directly to the plasmalemma. On top of that a much thicker (about 0.5 μm) layer is attached which consists of a complex three-dimensional array of soluble plasma components possibly including a variety of proteins and soluble glycosaminoglycans. This layer is in a dynamic equilibrium with the flowing plasma. Therefore, the thickness and composition of the surface layer depend on the plasma composition and the local haemodynamic conditions.
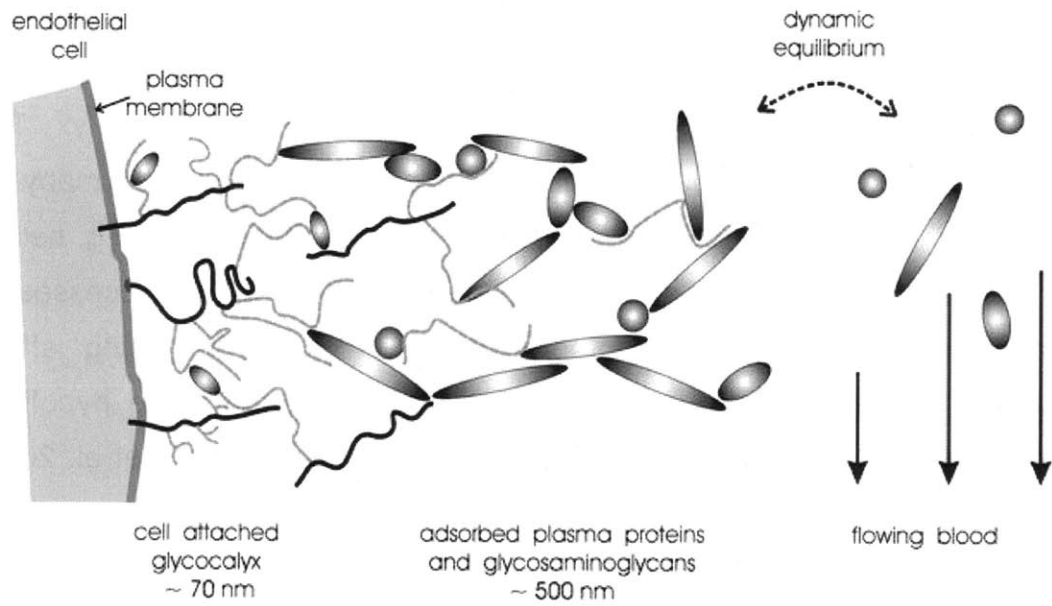
Fig5. A hypothetical composition of glycocalyx by A.R.Pries. About 0.5 um thick glycocalyx is attached to the cell surface, and it is in dynamic equilibrium with the flowing plasma. (Pries, Secomb et al. 2000)

One of the major components of the glycocalyx of the most interest to us is the syndecan family proteoglycan. Figure 6 is a schematic representation of two proteoglycans. All proteoglycans have long glycosaminoglycan (GAG) side chanis attached to a core protein. The length of the GAG chains is around 80nm (around 200 sugar residues). Syndecan-1 and syndecan-4 are the two major core proteins, and they are especially important because they are known to be the only syndecans that have cytoplasmic tails that interact with F-actin. Therefore, the syndecans may possibly construct a signaling pathway that can transmit shear stress to the intracellular cytoskeleton. A detailed description of the interation between syndecans and other important signaling molecules is shown in Fig 7. Research data (Dull, Dinavahi et al. 2003) has shown heparan sulfate GAGs (HSGAG) and chondrointin sufate GAGs (CSGAG) are the dominant GAGs on endothelial cells. Thus, our work will be focusing on finding out whether these two GAGs may be involved in shear-induced signaling pathways and their relative importance.
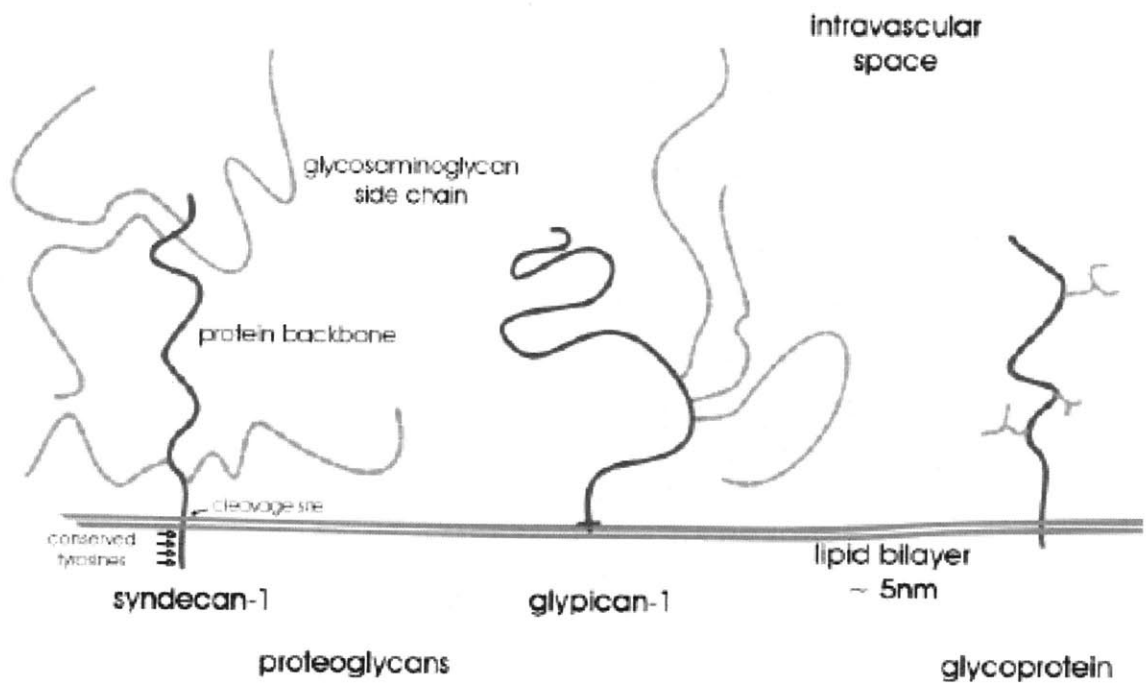
Fig6: Schematic representation of two types of proteoglycans on the endothelial surface belonging to the syndecan and glypican families, and a glycoprotein. The carbohydrate side chains of glycoproteins (e.g. selectins, integrins, members of the immunoglobulin super-family) are short and branched, while proteoglycans are characterized by long unbranched side-chains. (Pries, Secomb et al. 2000)

Fig7. Schematic representation of syndecan-4 acting as an organizing center for transmembrane receptors and is anchored to the actin cytoskeleton. (Bass and Humphries 2002)

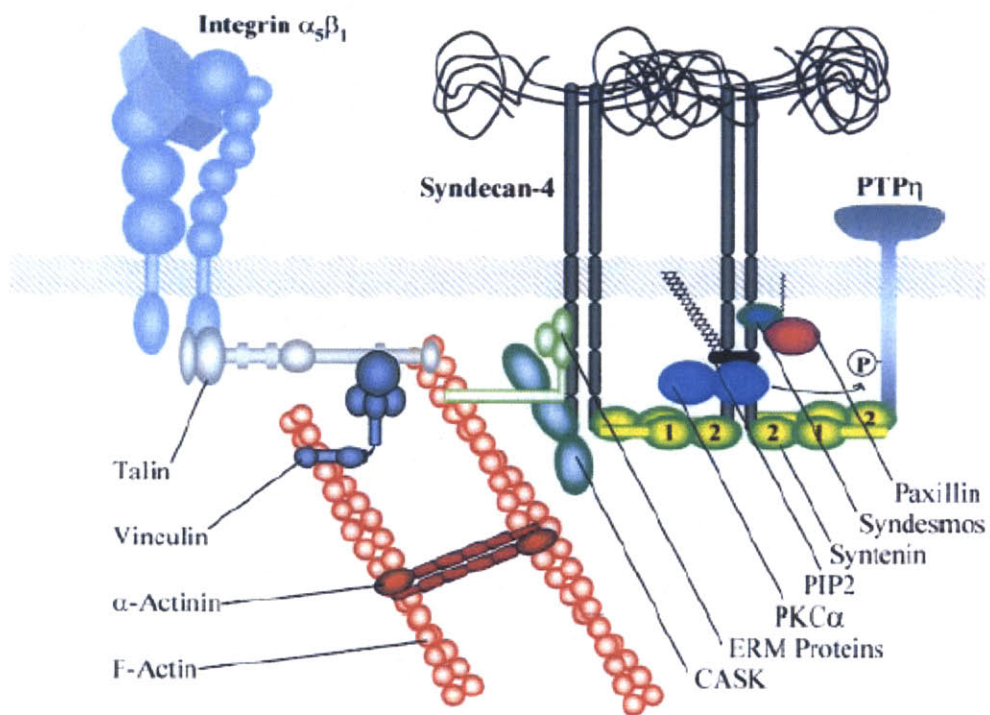A modified sketch of the structural model proposed by Squire(Squire, Chew et al. 2001) is shown in Figure 8 from the work of Weinbaum et al (Weinbaum, Zhang et al. 2003). The figure represents the core proteins in the proteoglycan clusters that comprise the glycocalyx and their linkage to the underlying actin cytoskeleton. 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 well as a 100-nm periodicity associated with the separation of each cluster and the observed hexagonal organization of the membrane bound foci.
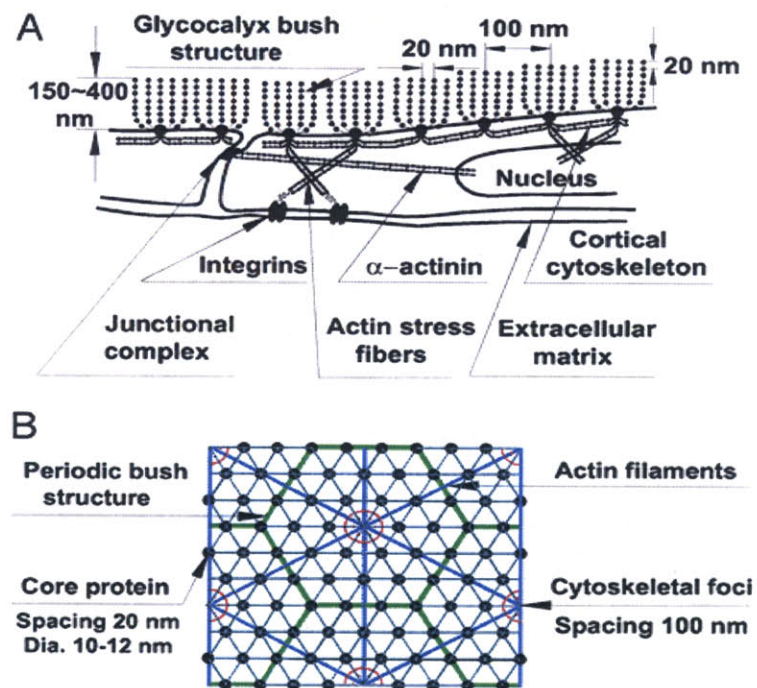

Fig 8. (A) Sketch of ESL (not to scale) showing core protein arrangement and spacing of scattering centers along core proteins and their relationship to actin cortical cytoskeleton (CC). (B) En face view of idealized model for core protein clusters and cluster foci and their relationship to hexagonal actin lattice in CC. (Weinbaum, Zhang et al. 2003)

1.2.3 Physiological implications of the glycocalyx

The glycocalyx is likely to have a strong influence on most interactions between flowing plasma and endothelial cell, and therefore to affect many physiological processes. So far, the investigation of potential functions of the glycocalyx has just been started, yet most of the work reported is still purely hypothetical. Based on the review by A.R.Pries (Pries, Secomb et al. 2000), some of these physiological functions are summarized as follows:

1. Flow resistance

The existence of the glycocalyx can be assumed to increase flow resistance in capillaries since it interferes with the free plasma flow. Observations of blood flow in microvessels (Pries, Secomb et al. 1990; Pries, Secomb et al. 1994; Pries, Secomb et al. 1997) implied that resistance in microvessels was higher than expected values based on the rheological behavior of blood in glass tubes, which can be attributed to the presence of the glycocalyx. This was also proved by direct measurement of flow resistence(Pries, Secomb et al. 1994) to blood flow in microvessels. Therefore, the presence of the glycocalyx has been shown to add additional energy cost to the circulatory system for a given vascular network structure.

2. Shear stress on endothelial cells

The fluid shear stress acting on endothelial cells needs to be transmitted to the cell and through the cell to the substrate. Since the fluidic property of membrane, it can not sustain the shear stress. It is reasonable to consider that the fluid shear is transmitted across the whole glycocalyx structure to intracellular cytoskeleton. The work done by Arisaka and coworkers (Arisaka, Mitsumata et al. 1995) showed that 24 hours of successive flow on endothelial cells can increase the amount of synthetic GAGs by cells.

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3. Regulation of blood flow

The calculations done by A.R.Pries (Pries, Secomb et al. 2000) indicated that higher lever of shear stress may cause the glycoclayx layer to be flattened against the cell surface, thus reducing the flow resistance to blood flow under high flow condition. This might explain the fact that skeletal muscle can modulate their blood flow 30 times faster during exercise compared to rest condition. Still, the concept has yet to be verified experimentally.

4. Barrier function

Extracellular, membrane-bound macromolecules have long been considered to play an important role in the transport of materials across the endothelium. Based on the fact that the glycocalyx is a negatively charged layer (negative charge from GAGs), several electro-diffusion models have been proposed to analyze various molecule transports across endothelium(Fu, Chen et al. 2003; Chen and Fu 2004). Vink and his colleagues (Vink and Duling 2000) demonstrated neutral tracers with molecular weights ranging from 0.4 to 40kDa permeate into the layer with halftime on the scale of 1min. While for anionic tracers as well as for fibrinogen (340 kDa) and albumin (67 kDa) permeation was much slower with halftimes between 11 and 60 min.

5. Coagulation

The endothelial surface layer plays a major role in the regulation of coagulation. Binding of antithrombin III to HSGAGs (ref) in the glycocalyx strongly affects the anticoagulatory properties of the endothelium. Antithrombin forms complexes with all serine proteases (with exception of factor VII)(Marcum and Rosenberg 1987; Mertens, Cassiman et al. 1992) of the coagulation pathway and thus interacts with the coagulation cascade at various levels.

6. Angiogenesis

Several studies show that HSGAGs in the glycocalyx can bind growth factors, angiogenetic factors and chemokines (Andres, DeFalcis et al. 1992; Basilico and

Moscatelli 1992; Gitay-Goren, Soker et al. 1992; Witt and Lander 1994). This indicates that the glycocalyx may play an important role in the modulation of angiogenesis. Studies of capillary glycocalyx in skeletal muscle (Brown, Egginton et al. 1996) provided evidence of glycocalyx involved in angiogenetic processes. After continuous electrical stimulation via implanted electrodes for 2-4 days, the capillary glycocalyx was observed to be disrupted or absent in a very high portion of capillaries.

7. Leukocyte adhesion

The concept of a thick macromolecular layer on the endothelial surface raised the question of how leukocytes adhere to the endothelium and how these receptors and ligands shorter than 100nm may interact (Dransfield, Cabanas et al. 1992). Weinbaum et al proposed models to predict the contact forces that are generated when a WBC with protruding microvilli rolls on a planar surface in a gravitational field, and estimated the length that microvilli can penetrate into the glycocalyx (Zhao, Chien et al. 2001). Marks et.al proposed that a WBC can tip-toe across the glycocalyx much like a Jesus Christ lizard can run across water (Marks, Stowell et al. 2001).

1.2.4 Recent research on glycocalyx

The location of the glycocalyx makes it logical to consider the role that glycoalyx can play under fluid shear stress. Recent investigators have show several results indicating glycocalyx can serve as a mechanosensor of fluid shear. Enzyme treatments were used to remove glycocalyx, and degraded cells were exposed to the flow and compared with normal cells. Florian et al (Florian, Kosky et al. 2003) reported that hepIII treatment can prohibit cultured endothelial cells' nitric oxide (NO) production in response to shear. Mochizuki et al (Mochizuki, Vink et al. 2003)also reported that hyaluronidase treatment can inhibit about 80% of NO production in response to shear. Furthermore, Thi et al (Thi, Tarbell et al. 2004) reported that digestion of glycocalyx can affect actin cytoskeleton reorganization,

redistribution of focal adhesion and cell-cell junctions. All these evidences encouraged our interest in studying how glycocalyx may affect shear-induced response of endothelial cells.

CHAPTER 2. Degradation of the glycocalyx

To study whether the glycocalyx is involved in the shear-induced signaling pathway, we use specific enzymes for partial degradation of the glycocalyx, and then compare the behavior of these degraded cells to normal cells. HSGAGs and CSGAGs are known to be the dominant GAGs on glycocalyx, and they are attached to syndecans that interact with F-actin. Therefore, we use two different enzymes specifically targeted to these two GAG components. By fluorescent labling of GAGs, we can estimate to which extent these GAGs can be removed.

2.1 Materials and methods

2.1.1 Cell culture

Bovine aortic endothelial cells (BAEC) were used in all these experiments. BAECs from passage 5 to passage 15 were cultured on gelatin coated (0.2%) T75 flasks. Cells were grown in DMEM, supplemented with 1% penicillin/streptomycin, 10% fetal bovine serum (FBS) and 1% glutamine. For experimental work, cells were seeded on 25X75mm cover slips. All cells were kept in an incubator with maintained environment at 37° temperature and 5% CO_2.

2.1.2 Microscope and fluorescent labling of heparan sulfate GAGs

All images during experiment were obtained with a Nikon Eclipse T2000 inverted microscope using 10X, 40X or 60X objectives and an Apogee KX32ME CCD camera thermoelectrically cooled to -10° C.

To image the heparan sulfate GAGs, the EC monolayers were first washed with DPBS, and a solution of 2μL HepSS-1[31] was diluted to 1ml using DPBS and applied to the monolayers. After a 15-minute incubation period, 2μL Alexa Fluor 488 labled secondary antibody was diluted to 1ml with DPBS and applied to the

monolayers for another 15-minute period. Then the monolayers were washed twice with DPBS and imaged with fluorescence using a 60X objective on the microscope with an exposure of 750ms. Comparison of the fluorescence intensity of the untreated and treated imaged fields provided an estimate of the degree of heparan sulfate removal using heparinase treatment.

2.1.3 Enzyme treatment of the glycocalyx

Heparinase treatment

Heparinase III is an enzyme selective for heparan sulfate within the glycocalyx. According to published data (Dull, Dinavahi et al. 2003), hepparinase III can remove about 70% of heparan sulfate GAG. For heparinase treatment, the monolayers were washed with serum free medium twice, and 200mU heparinase solution were placed on the monolayers and incubated in 37° and 5% CO_2 for 30 min before experiments. After incubation, the monolayers were washed with DPBS twice, and supplied with growth medium before experiments.

Chondroitinase ABC treatment

Chondroitinase ABC is an enzyme selective for chondroitin sulfate within the glycocalyx. For chondroitinase treatment, the monolayers were washed with serum free medium twice, and incubated with 200mU chondrointinase for 30 min. After incubation, the monolayers were washed with DPBS twice, and supplied with growth medium before experiments.

2.2 Results

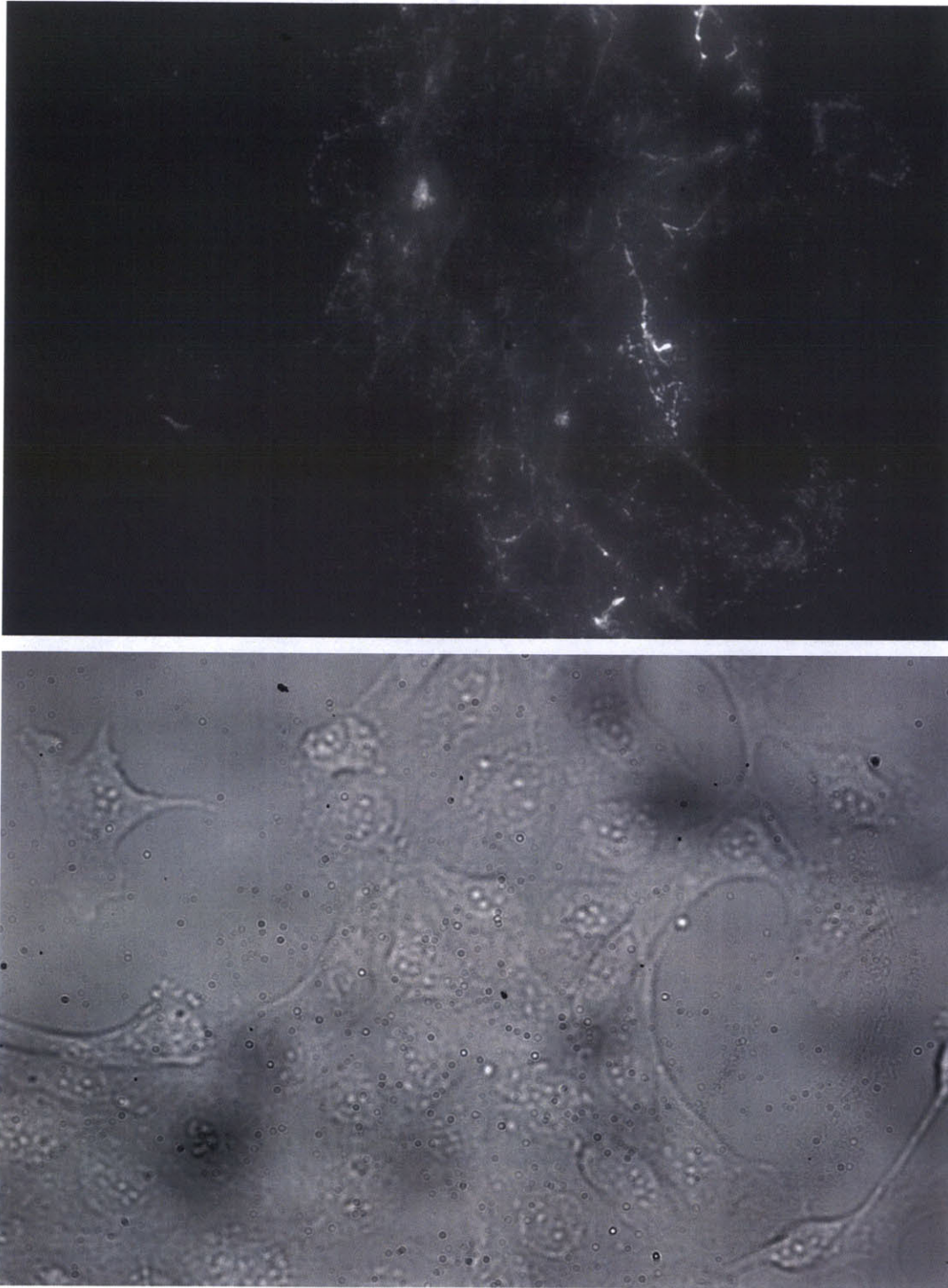Fluorescent images of heparan sulfate are shown below.

Fig 9. Fluorescence image of heparan sulfate on a patch of normal ECs.
Top: fluorescent image. Bottom: bright field image of the same patch of ECs.
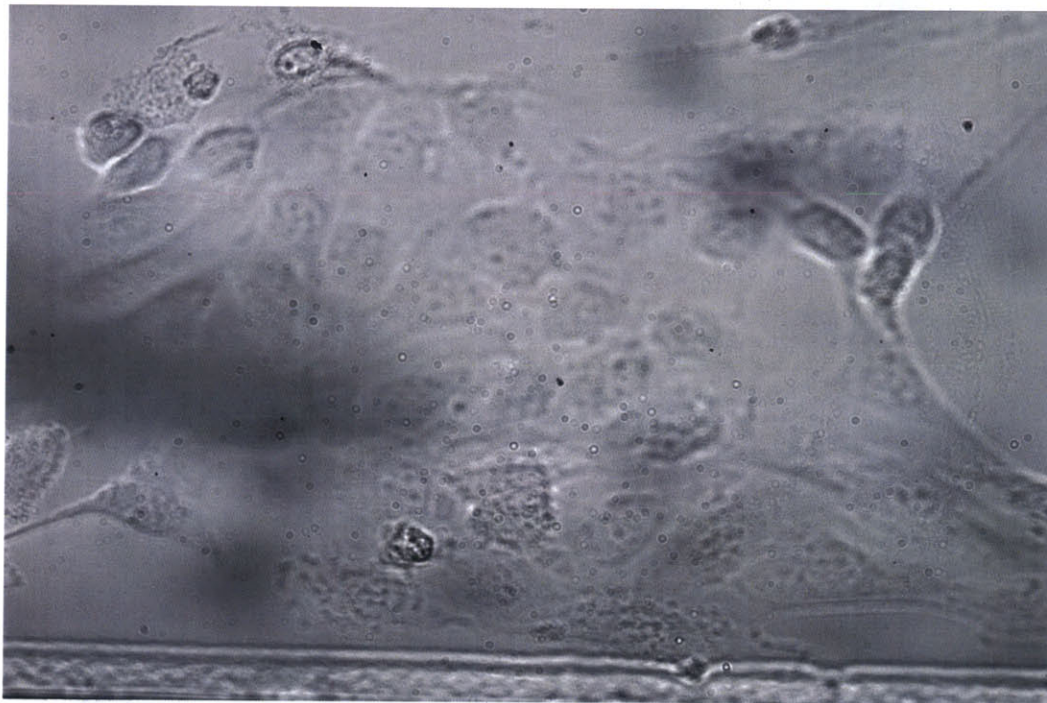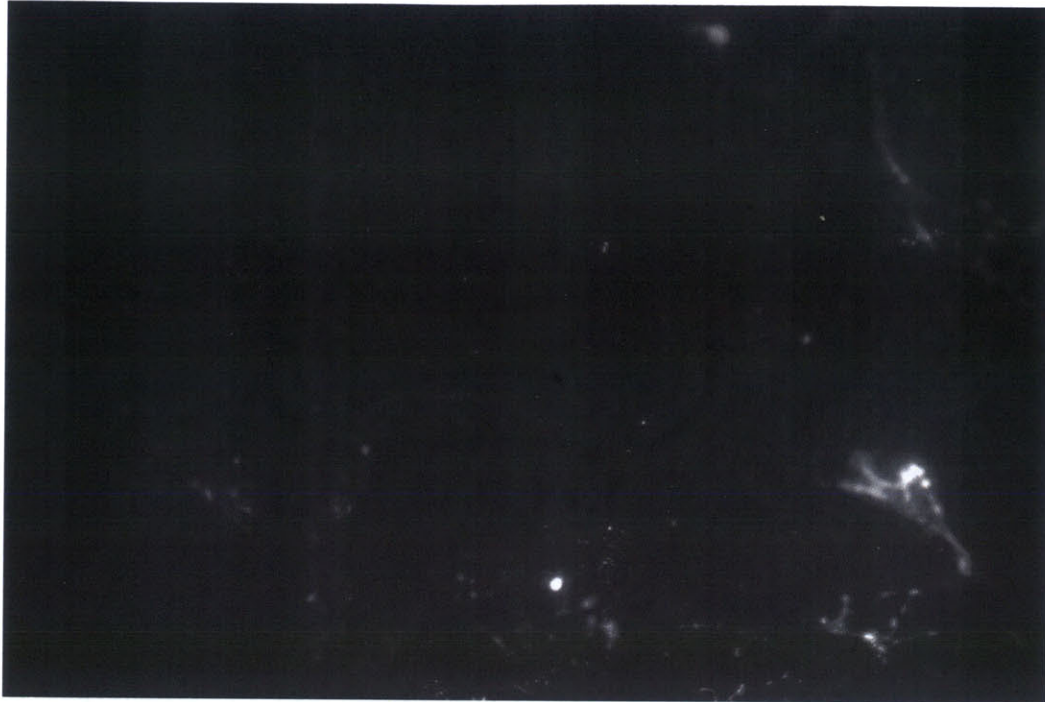
Fig 10. Fluorescent image on a patch of heparinase degraded cells.
Top: fluorescent image. Bottom: bright field image of the same patch of ECs.
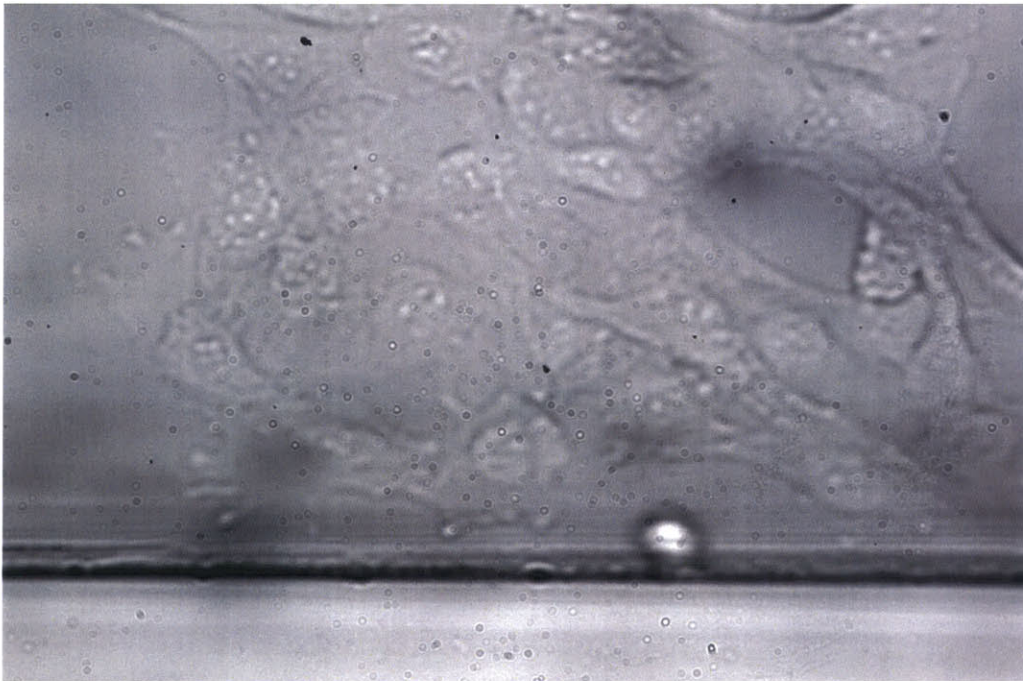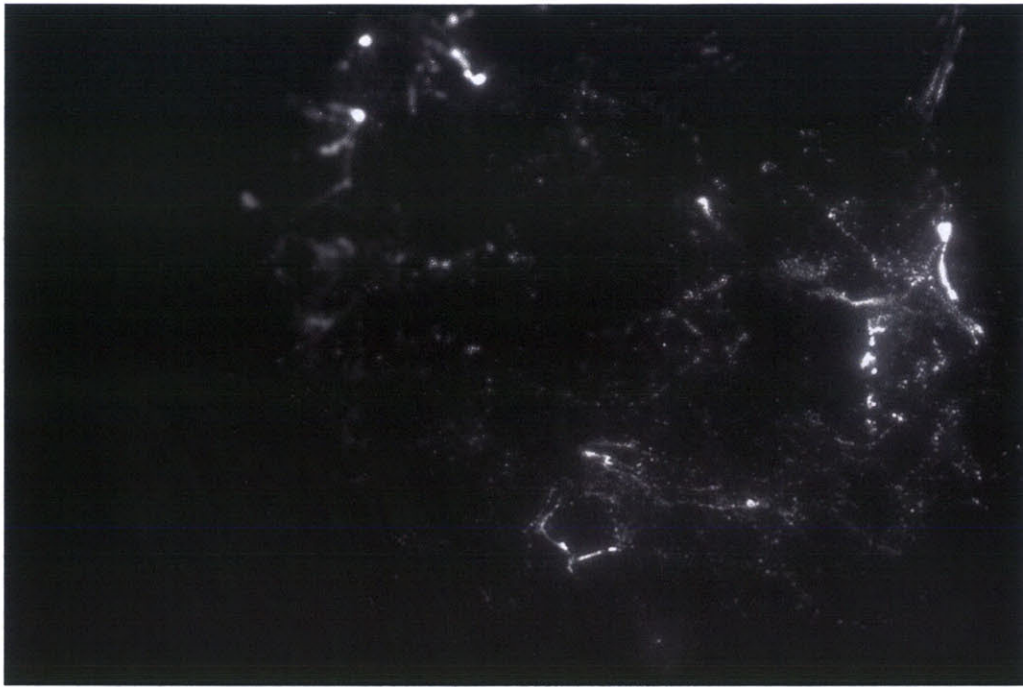
Fig 11. Fluorescent image on a patch of chondroitinase degraded cells.
Top: fluorescent image. Bottom: bright field image of the same patch of ECs.

By comparing the fluorescent intensity of heparan sulfate on normal cells, heparinase degraded cells, and chondroinase degraded cells, we can see that a significant amount of heparan sulfate were removed by heparinase treatment, while chondrointinase treatment didn't affect the heparan sulfate distribution.

CHAPTER 3. Comparison of degraded ECs behavior to normal ECs under static condition

3.1 Experiment design
3.1.1 Crawling speed experiment

To examine degraded ECs' behavior at static condition, we start with cell crawling speed measurement. Bright field images of a specific region of an EC monolayer were taken under microscope every 5 min. Each one-hour experiment yields 13 images. About 40 cells were randomly picked for each experiment, and their nucleus positions were tracked on each image using Image J software. The crawling speed of each cell was calculated based on the length of cell trajectories, and the mean speed of each experiment was from the statistics of 40 cells.

3.1.2 Wound healing experiment

We used a 200ul pipette tip to scratch the surface of an EC monolayer, and calculate the wound width right after the scratch. After the monolayer was kept in the incubator for 4 hours, the wound width was calculated again. By dividing the change of wound width by 4, we got the average wound recovery speed. Comparing the average wound recovery speed between normal cells, heparinase degraded cells and chondrointinase degraded cells can give us additional information on cell migration.

3.2 Results

3.2.1 Crawling speed experiment

We've detected an average of 30% crawling speed drop right after the heparinase treatment, which indicates heparinase treatment alone can change ECs' mobility. However, ECs' crawling speed only dropped about 15% after chondoitinase treatment. Then we measured degraded cells crawling speed at 1st hr, 8th hr and 19th hr after treatment, and the associated data showed the recovery of crawling speed with respect to time, which is about the same time scale of glycocalyx recovery. (unpublished results from David Berry)
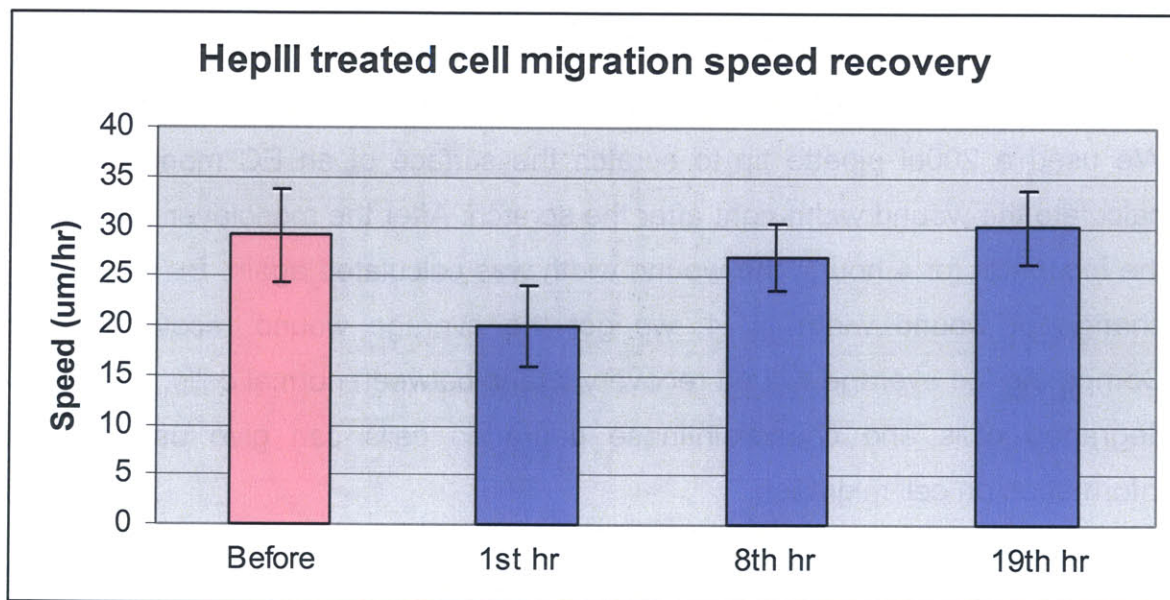

Fig 12. HepIII treated cell crawling speed recovery over 19hrs.
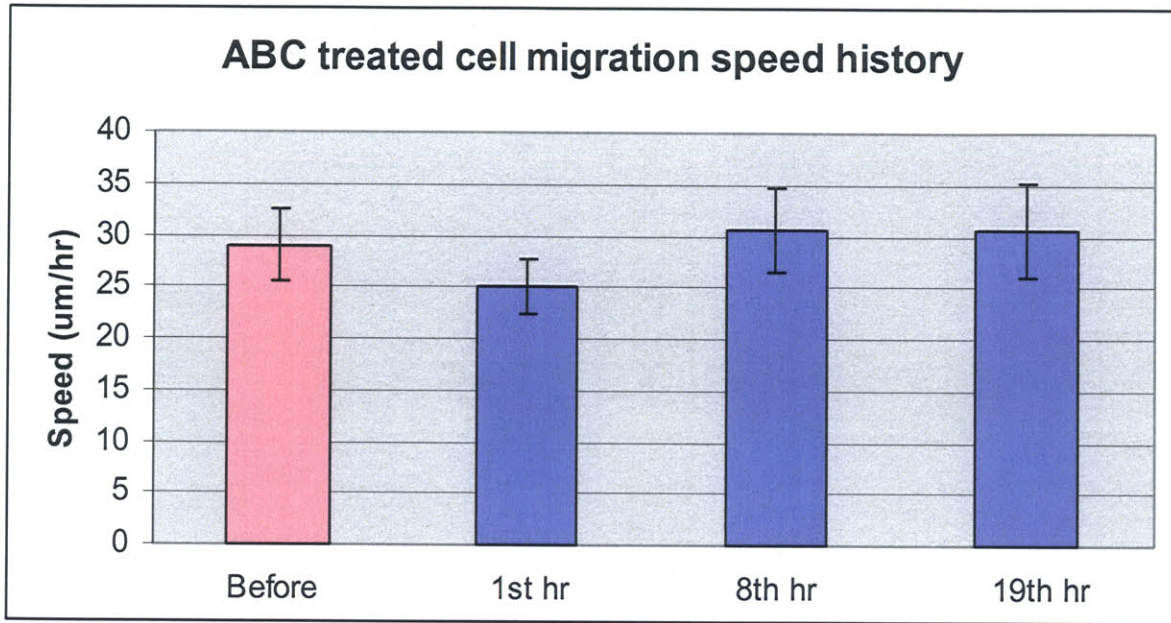
Fig 13. Chondroitinase ABC treaded cell recovery over 19hrs.

3.2.2 Wound healing experiment

During wound healing experiment, we've detected a significant recovery of monolayer during 4 hours. Wound healing speed is calculated according to the change of the wound width divided by 4 hours. In addition, calculation of average wound healing speed of 3 cell types has shown strong consistence with the crawling speed of these cells at static condition.

Fig 14. Normal BAEC wound edge recovery in 4 hours.
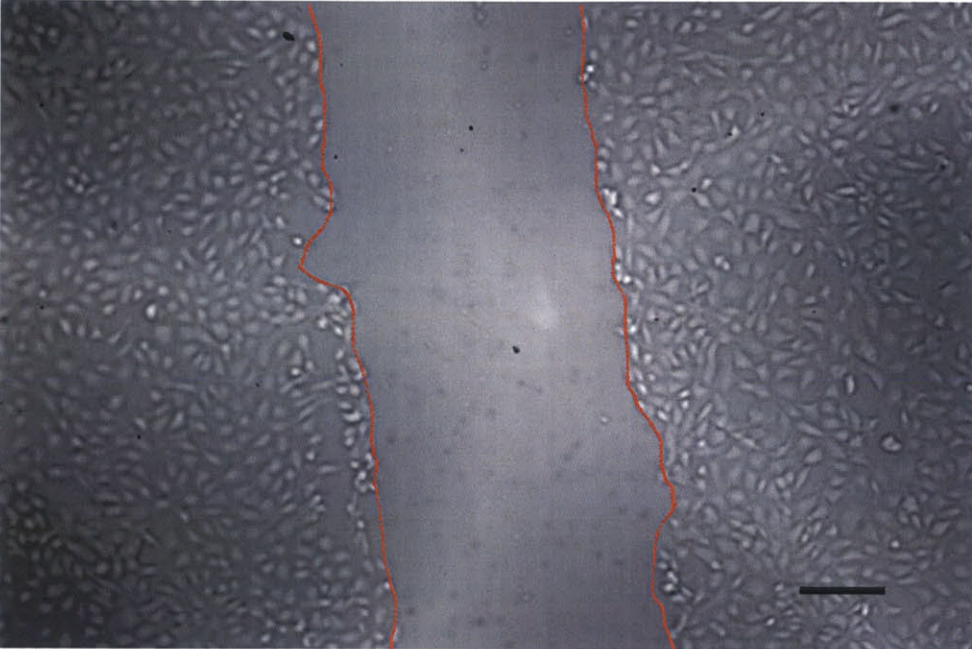
Average wound width about 169um after scratch of surface.
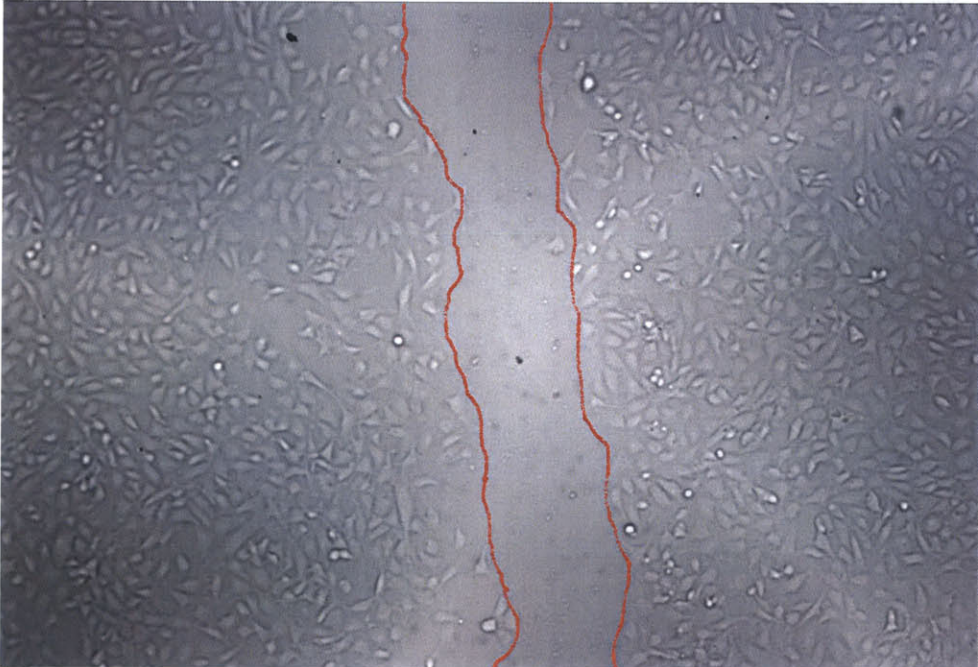
Average wound width about 78um 4 hours later.

Wound recovery speed about 22.7um/hr

Scale bar 50um

Fig 15. Heparinase degraded BAEC wound edge recovery in 4 hours.

Average wound width about 223um after scratch of surface.
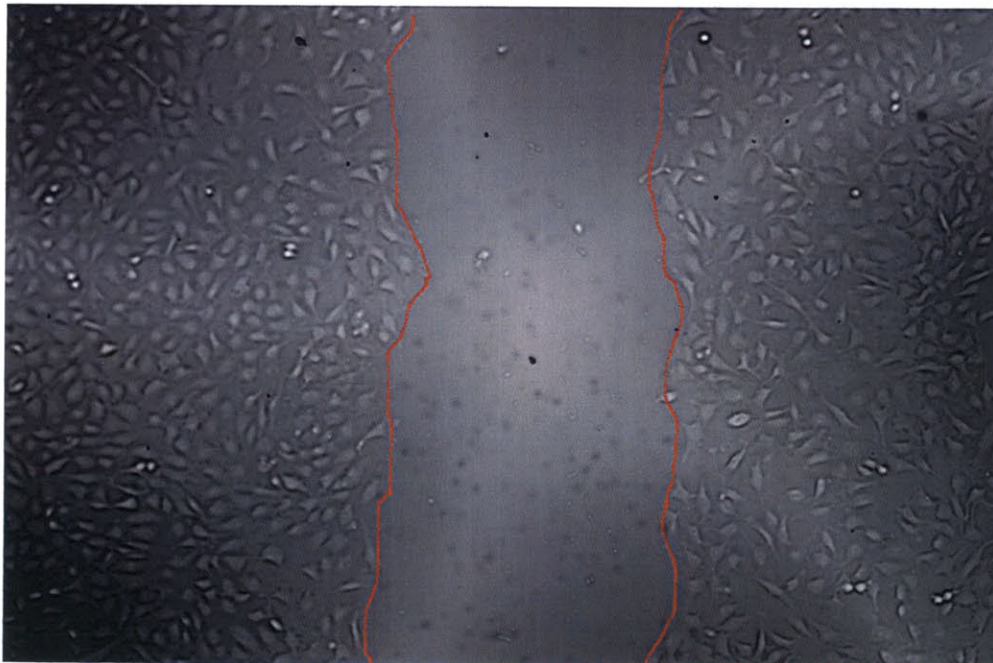
Average wound width about 165um 4hours later.

Wound recovery speed about 14.5um/hr

Scale bar 50um

Fig16.Chondroitinase degraded BAEC wound edge recovery in 4 hours.
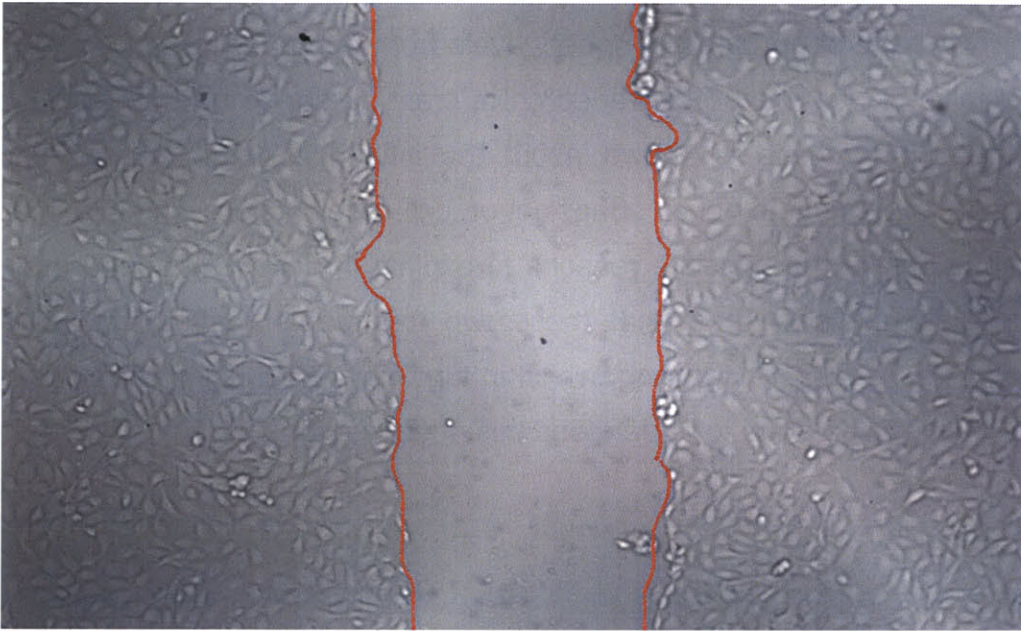
Average wound width about 157um after scratch of surface.
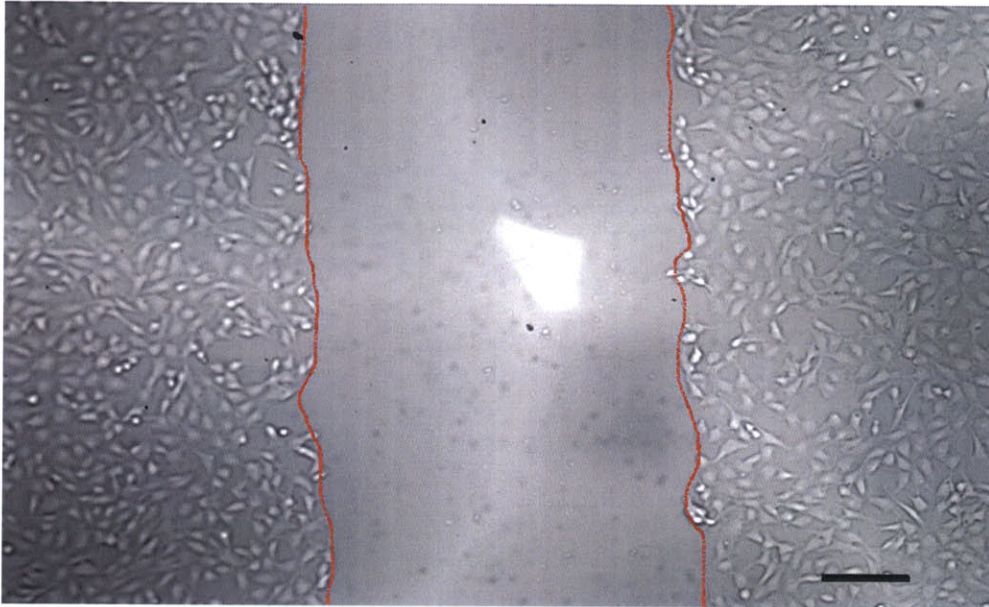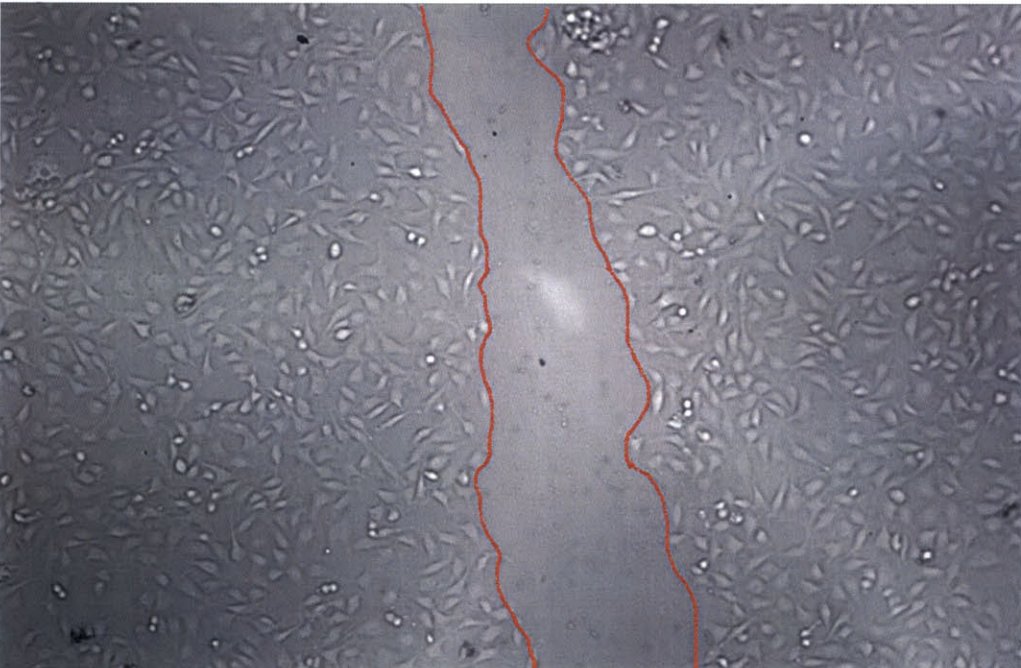

Average wound width about 84um 4 hours later.

Wound recovery speed about 18.5um/hr

3.3 Discussion

Based on the results from both crawling speed experiment and wound healing experiment, we have seen different speed levels from degraded cells to normal cells. Especially for heparinase degraded cells, crawling speed had an average drop of 30%. So far, little is known about the influence of heparinase and chondroitinase treatment to focal adhesion or cell-cell junction, and we may assume this is one of the possible reasons to explain the crawling speed drop. Another possible explanation is that these two treatments may affect some signaling pathway, e.g. Ca^{2+} signaling, which in turn may affect cell mobility. Still, more study needs to be done to understand this phenomenon.

CHAPTER 4. Comparison of flow-induced response of degraded ECs to normal ECs

As stated before, endothelial cells respond to flow both in short term and long term. Since glycocalyx is right on top of the ECs, where fluid shear stress is exerted on, by removing the glycocalyx and measuring the degraded cells response to shear may provide our insights to better understanding of the shear response mechanism.

4.1 Experiment design

4.1.1 Flow chamber

A parallel-plate flow chamber is used to subject culture BAEC monolayers to laminar fluid shear stress. The chamber (Figure 17) is composed of two acrylic plates separated by a piece of silicon sheeting. The flow chamber is formed by removal of a 1cm x 15cm rectangular section from the gasket. The channel height is defined by the thickness of the gasket, which is 0.75mm. A single cover slip with confluent BAEC monolayer is placed in a rectangular recess in the bottom plate, and a region of the monolayer is exposed to flow (10mm x 75mm). The chamber is maintained at $37°$ by blowing hot air into a cage that covers the chamber.

4.1.2 Flow system

The whole flow system is composed of one flow chamber, one fluid reservoir, one flow pump, one dampener, and several connecting tubing (Figure 17). The flow generate by the pump circulates through the damper to the flow chamber and back into the reservoir. The damper is sealed so that there will be a constant pressure drop across the flow chamber soon after we start the flow. This constant pressure drop yields a stable shear stress on the cells. The fluid reservoir is filled with growth medium and heated in the water bath to $37°$. Meanwhile, it is supplied with a gas mixture of 5% CO^2 and 95% of air as its environment.

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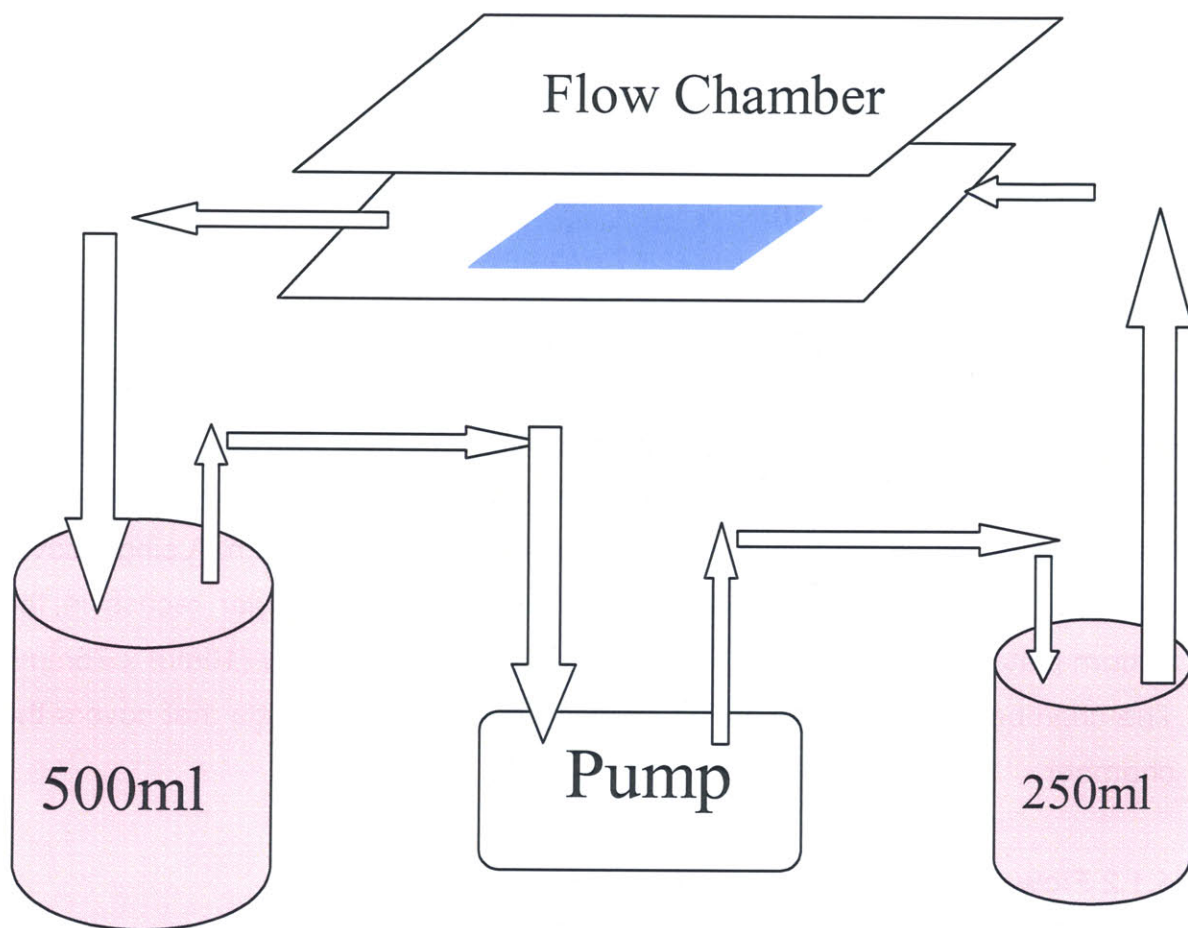
Fig 17. Flow system setup. Whole system includes one flow chamber, one pump, one fluid reservoir, one damper and connecting tubing.

4.1.3 Shear stress calculation

The channel flow can be approximated as two-dimensional fully developed laminar flow with a simple parabolic velocity profile, since the channel height is much less than the width. Thus, shear stress at the wall can be calculated using

$$\tau = \frac{6\mu Q}{h^2 b}$$

the flowing formula:

Flow rate Q: 80ml/min

Fluid viscosity u: 10^3 kg/ms

Channel width b: 1cm

Channel height h: 0.075cm

By applying a constant flow rate of 80ml/min, we have a shear stress of 15 dyne/cm^2.

4.1.4 Crawling speed experiment

It has been observed for many times (unpublished results by Osborn) that shear stress can lead to a significant drop of crawling speed during the first couple of hours when we apply the flow, though the mechanism is still not well understood. Thus, cell crawling speed can be a good indicator of shear-induced response for our experiments. The way to measure the crawling speed is the same as described in Chapter2.

4.2 Results

4.2.1 Normal cell crawling speed

Normal conflent BAEC crawling speed under flow

Start flow

speed (um/hr)

Before Flow   1st hr   2nd hr   3rd hr   4th hr
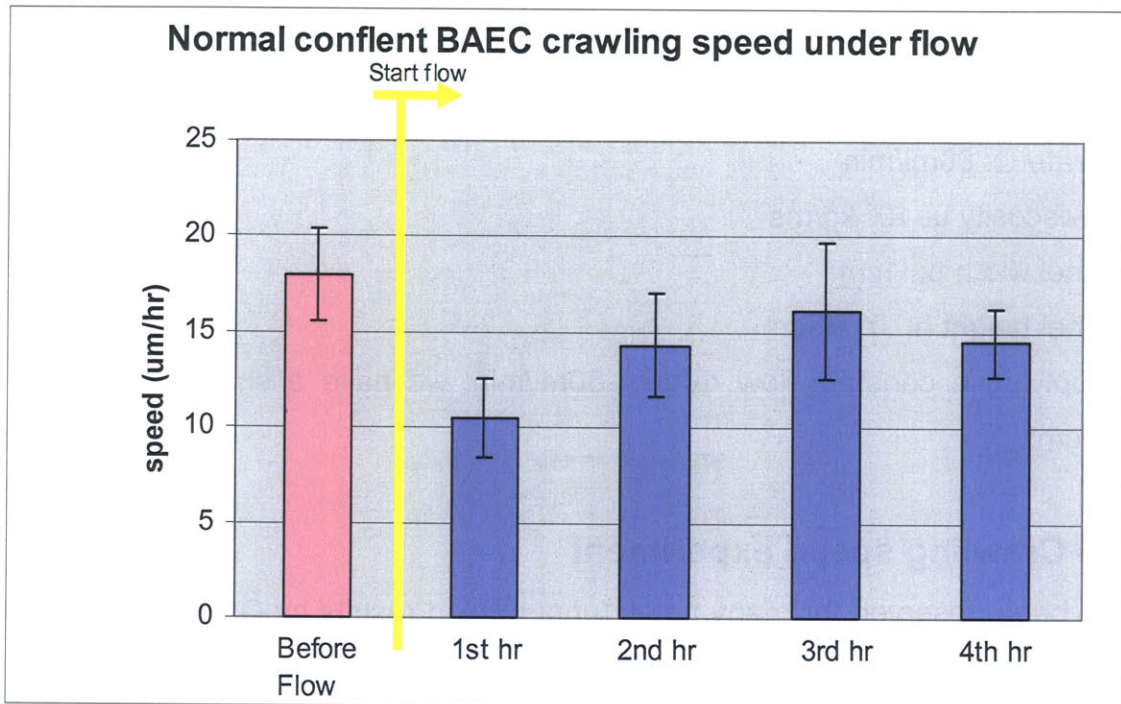
Fig 18. Normal confluent BAEC crawling speed before and after the flow. The crawling speed has a significant drop in the first couple of hours after the flow.

4.2.2 Heparinase degraded cell crawling speed

HepIII treated cell migration speed under flow
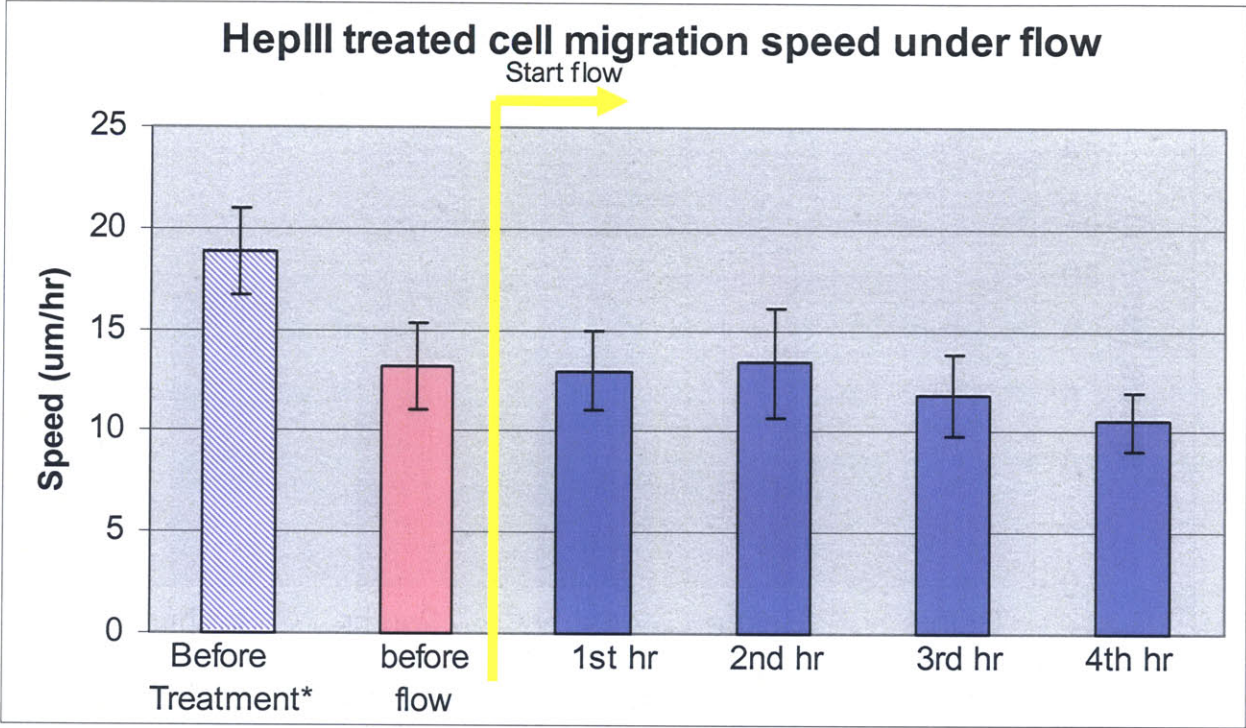
Fig 19. HepIII treated BAEC crawling speed before and after the flow. The data labeled as before treatment is an estimate of normal cell crawling speed without flow based on previous statistical data. There is no significant drop of crawling speed after the application of the flow compared

4.2.3 Chondroitinase degraded cell crawling speed

Fig 20. Chondroitinase treated BAEC crawling speed before and after the flow. The data labeled as before treatment is an estimate of normal cell crawling speed without flow based on previous statistical data. The data shows a significant drop of crawling speed after the application of flow.

4.3 Discussion

From the data, we can see that HepIII degraded cells do not respond to the flow in terms of crawling speed, while Chondroitinase degraded cells respond to the flow quite the same as the normal cells. There are a few possible reasons to explain this.

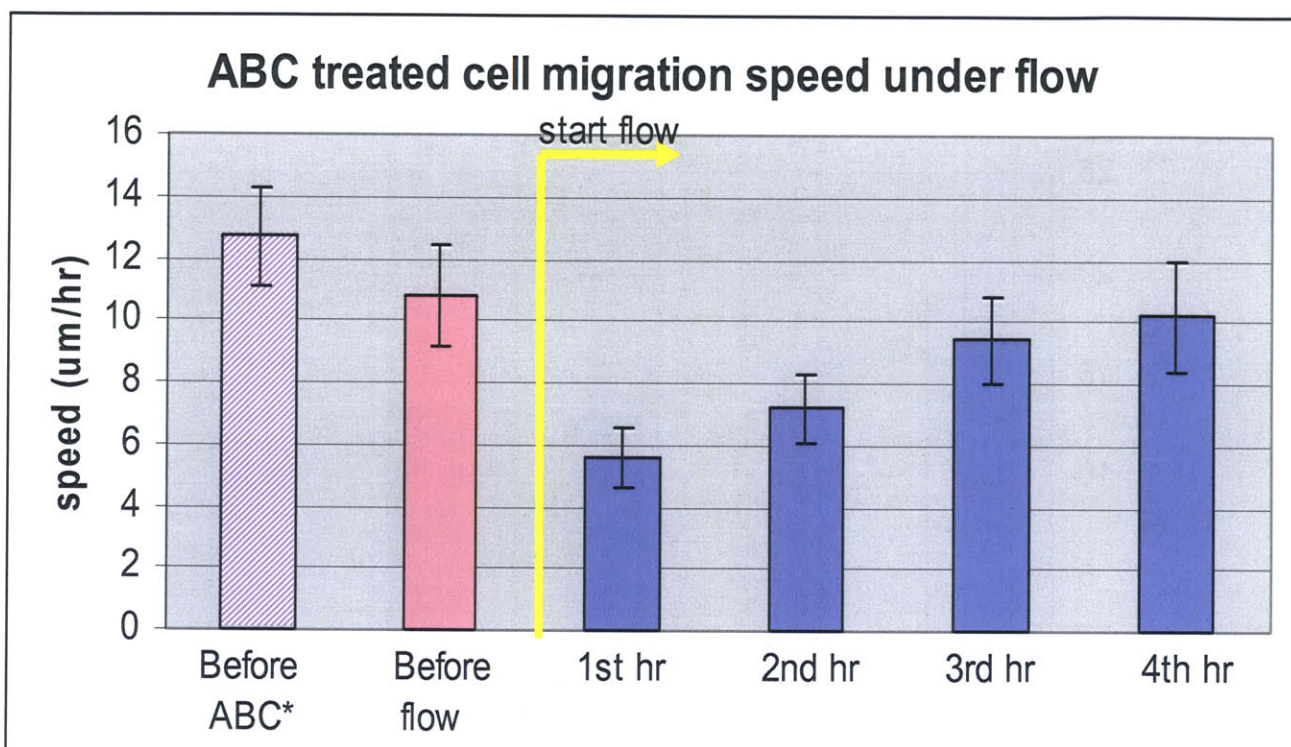(1) The amount of heparan sulfate GAGs could be much more than chondroitin sulfate GAGs in the glycocalyx, so that the glycocalyx structure collapses when using HepIII to remove its dominant GAGs, while using Chondroitinase has small effect on the integrity of the whole structure.

(2) There could be much greater percentage of heparan sulfate GAGs linked to syndecans, while the majority of the chondroitin sulfate GAGs are associated with other non-syndecan family proteoglycans. Due to the fact that syndecans are the only known proteoglycans that interact with actin cytoskeleton (REF), it is reasonable to believe that only those GAGs associated with syndecans can be considered as functional GAGs in terms of flow response.

(3) It is also possible that the heparan sulfate GAG chain on the endothelial surface is longer than chondroitin sulfate GAG, thus giving rise to a greater torque exerted on the cell when shear stress is applied. However, the mechanisms determining the GAG length are still unknown (Lodish, Berk et al. 2000).

(4) Heparan sulfate GAG is known to be involved in many physiological processes (REF). For instance, heparan sulfate GAG is needed to bind growth factors and help them interact with growth factor receptors (Lodish, Berk et al. 2000). Therefore, it might be possible that the heparan sulfate could somehow affect the transportation of some signaling molecules that are involved in the shear-induced response. Moreover, the heparan sulfate GAG is the only type of the GAGs that can have different degrees of sulfation on the chain that may give them more

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charge density than chondroitin sulfate GAG since it is the sulfate group on the chain that bears negative charge. In that case, more charge density can give rise to more electric bulging effect in the whole structure.

Chapter 5. Conclusion and Future work

5.1 Conclusion

In this thesis, we started investigating the effect of glycocalyx in the shear-induced cellular response by monitoring crawling speed and wound recovery speed either at static condition or under the application of flow. From our preliminary results, we have come to the conclusion that the existence of the glycocalyx is involved in the pathway that endothelial cells sense and respond to the flow, and by removing part of the glycocalyx, endothelial cells' crawling speed response to the flow is highly suppressed. However, endothelial cell crawling speed can be affected by several factors, such as focal adhesion and cell-cell junction; yet whether one or even more of these factors may be somehow influenced by removing glycocalyx is still not clear. Meanwhile, there are two possible mechanisms that the glycocalyx can be involved in mechanotransduction: one will be the biomechanical way that the forced exerted on top of the glycocalyx is transmitted into the intracellular space, and therefore induce the actin cytoskeleton reorganization; the other will be the biochemical way that the existence of the glycocalyx will be important for the diffusion of some signaling molecules, and removing the glygocalyx can affect the interaction between these molecules and their receptors. More research need to be done for better understanding of the importance of the glycocalyx in the mechanotransduction, and this also provides great challenge for our future work.

5.2 Future work

Our currents results have provided us confidence to continue on our current research project on the glycocalyx, and more can be done to prove the involvement of the glycocalyx in the shear-induced mechanotransduction. Therefore, we are planning the flowing experiments in the near future.

1) Since the early 90's, researchers have found that cytoplasmic calcium concentration has a sharp increase within minute after the application of

flow (Shen, Luscinskas et al. 1992), and calcium is an important secondary messenger that many cellular responses occur in a calcium dependent pathway. For example, many actin-binding proteins (e.g. gelsolin) are calcium dependent as well as some cell-cell juctional molecules (VE-cadherin). Thus, we can examine whether the calcium response can be affected by changing the state of the glycocalyx.

2) Recent work in our lab is investigating VE-cadherin as an important juctional molecule that the short-term response of the EC crawling speed may be explained by the diffusion and clustering of the VE-cadherin at the cell borders. As we know, VE-cadherin is a calcium dependent molecule, and if calcium response can be affected by changing the state of the glycocalyx, we may expect that the dynamic response of the VE-cadherin may also be affected.

3) Actin cytoskeleton starts after the application of the flow. Recent work (unpublished results by Osborn and others 2005) has shown that the actin turn over increases within 30 min of the flow, and the fraction of polymerized actin decreases at the same time, which indicates the remodeling of the cytoskeleton. Therefore, we may examine the effect of the glycocalyx on the actin reorganization by using PAF/FRAP experiments (McGrath et al 1998).

4) Syndecan is the one of the core proteins where the GAGs are linked and it is the only one that has a cytoplasmic tail that ineteract with F-actin (Bass and Humphries 2002). If the glyococalyx is involved in the force-transmission way of shear-induced response, then syndecan should serve as a critical protein to complete this force-transmission pathway. By blocking syndecan and looking at the cellular response to flow, we may get information on whether syndecan is involved in this pathway.

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