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Effect of shear stress on macromolecule uptake in porcine aortic endothelial cells *in vitro*

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Title (English)

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Title (Swedish)

Abstract

An *in vitro* model for studies of the shear stress response of endothelial cells was developed. Using this model, the effect of shear stess on endocytosis of potentially harmful molecules such as lectins and low density lipoproteins were studied using confocal microscopy. The effect of heparane sulfate proteoglycan on this transport was also investigated.

Keywords

Endothelial cells, HSPG, shear stress, glycocalyx, endocytosis.

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Sammanfattning

Att det finns ett samband mellan blodflöde (turbulent eller laminellt) och utveckling av ateroskleros (åderförkalkning) är väl etablerat. Det är dock inte helt kartlagt på vilket sätt endotelceller påverkas av blodflödet eller vad som gör att kärlväggarna i blodkärlsförgreningar och bifurkationer som upplever stört flöde har en större tendens att utveckla aterosklerotiska plack. Den kraft som blodkärlsväggarna upplever från det flödande blodet kallas skjuvspänning. På senare tid har fokus lagts på att undersöka vad glycokalyx, det proteoglykanrika ytlagret på endotelcellerna, har för roll i cellernas svar på flöde.

En viktig del i utvecklingen av ateroskleros är deposition av lipoproteiner, släta muskelceller, proteoglykaner och bindväv i kärlväggen. Detta examensarbete fokuserar på heparan sulfat proteoglykaner (HSPG) som är en viktig komponent i glykokalyx och på sambandet mellan förekomsten av HSPG på cellytan, skjuvspänning och cellulärt upptag av HSPG specifika lektiner och lipoproteiner. I projektet ingår bland annat utveckling av ett *in vitro*-system där celler kan exponeras för en väl specificerad skjuvspänning under längre tidsperioder. Projektet är en del i ett flerårsprojekt med syfte att studera vad som händer i cellerna under ett tidigt stadium i aterosklerosutvecklingen bland annat genom att simulera flödet av makromolekyler och oxidanter över kärlväggen i olika flödesprofiler ”*in silico*” och genom att kartlägga effekten av glykokalyx på denna transport.

Examensarbete 20p
Civilingenjörsprogrammet i molekylär bioteknik

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INTRODUCTION

Hemodynamics and cardiovascular disease

Cardiovascular disease is the major cause of mortality worldwide but despite great efforts current strategies aimed at lowering the systematic risk factors have only achieved a 20-30% decrease of the disease. As the endothelium is the first line of defense against atherosclerosis much of the research in this area is focused on improving endothelial function.

Atherosclerosis is a disease of the coronary, carotid and other proximal arteries and involves a distinctive accumulation of low-density lipoproteins (LDL) and other lipid bearing materials in the arterial wall (Fig. 1). The importance of hemodynamics on endothelial cell response has been known since the early studies by Fry (1976) and Caro and Nerem (1973). It is well known that atherosclerosis appears as a focal inflammatory disease at arterial tree bifurcations, junctions and regions with high curvature (2). These observations and a host of *in vitro* experiments over the past 25 years have shown that mechanical stresses due to blood flow are of major pathophysiologic importance throughout the atherosclerotic disease process (3). In the relatively straight regions in the arteries with high hemodynamic shear stress (> 10 dynes/cm^2), the endothelial cells lining the vessel wall are elongated in the flow direction with relatively tight cell-cell junctions. These regions show much higher resistance to disease development compared to bifurcation regions or sections with high curvature where flow separation and recirculation result in low average shear stress or oscillatory shear index (Fig. 2) (4-13). Although much is known about the correlation between the hemodynamic stress and cell response, current understanding of the *mechanism* by which hemodynamic stress influences the endothelium is limited. Considerable effort is underway to better understand the underlying pathophysiology of atherosclerosis and the fundamental biology and biomechanics of atherosclerotic lesion. It is also important to understand the mechanism that calcification and lesion formation preferentially occurs in regions with 'disturbed' flow.

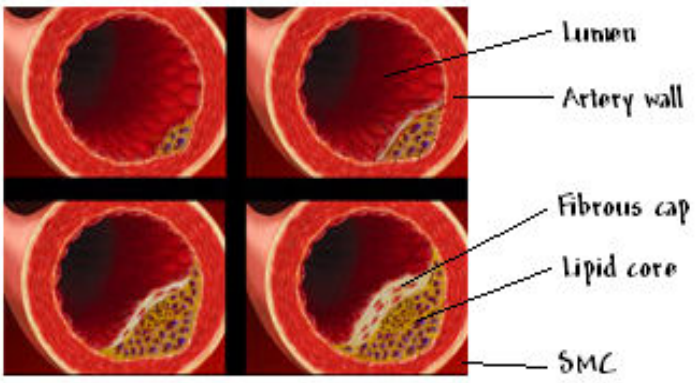


Figure 1. Cut section of artery showing the development of the atherosclerotic plaque in the vessel wall.

The project

Despite significant clinical advances, a better understanding of the underlying pathophysiology of atherosclerosis and the fundamental biology and biomechanics of atherosclerotic lesion is needed. Previous studies have shown that mechanical stresses due to blood flow are of major pathophysiological importance throughout the atherosclerotic disease process. More recent *in vitro* and *in vivo* studies point to the central role of the glycocalyxlayer (GL) as a mechanotransducer in hemodynamic shear stress-induced NO production. Combination of the recent progress in mutliscale modeling with the modified immunohistochemical (IHC) *en face* method based on quantum dot (Qdot) bioconjugates and two-photon excitation laser scanning microscopy (TPELSM) and gene expression analysis provides a unique and unprecedented opportunity to reveal the underlying pathophysiology of atherosclerosis. My project is part of a bigger collaboration with the ambitious aim to model the transport of substances across endothelium in vessel bifurcation, trying to elucidate what factors are crucial in the onset of atherosclerosis. My part of the project deals with the proteoglycan rich surface structure of endothelial cells known as the glycocalyx and the effect of shear stress on this structure and on the endocytosis of macromolecules such as LDL. One important aim is to create an *in vitro* model were cells can be exposed to specified amounts of shear stress and thus confirms the results predicted by the computer model in the future.

The glycocalyx

The studies of cell response to hemodynamic stress stimuli have been focused recently on the mechanotransduction from the extracellular layer to the interior region of the cell through the surface proteoglycan (glycocalyx) layer. The glycocalyx is an organized mesh of membranous glycoproteins, proteoglycans, glycosaminoglycans (GAGs) and associated plasma proteins, which can reach up to 3 μm into the vessel lumen (14). Except for its role as a mechanotransducer, the glycocalyx is believed to function as a molecular sieve, a hydrodynamic exclusion layer and to modulate leukocyte attachment

and rolling (15). The prevailing structural model suggests that proteoglycan core proteins make up the center while associated GAGs contribute to the branch-like structure giving glycocalyx its molecular sieving properties. (Fig. 4) Heparan sulfate (HS) is the most common GAG and is associated with 50 – 90% of the proteoglycans and it is present in a ratio of 1:4 with the second most common GAG, chondroitin (CS). Importantly, heparan sulfate proteoclycans (HSPG) also mediate interactions with many protein ligands, including growth factors, morphogens, and their receptors (46). HSPGs can further be divided into different groups based on their protein backbone. The most common HSPGs belong to the Syndecan family, the only components of the glycocalyx known to penetrate into the cytoplasm. Syndecan I is the most prevalent of this family of four members. Typically Syndecans in the endothelial glycocalyx have one to three HS and one to three CS GAG molecules attached to their core proteins (16,17). The GAG hyaluronan is also abundant in the glycocalyx but is not attached to a protein core. The function of this GAG is probably to increase the sieving properties of the glycocalyx (18).

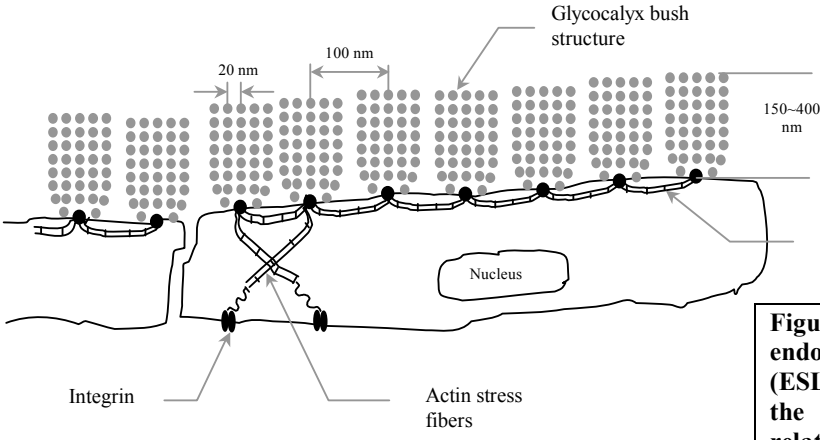


Figure 4. Schematic of the endothelial surface layer (ESL) showing a side view of the glycoproteins and their relationship to the cortical cytoskeleton. (15)

Studies in both micro and macro vasculature have demonstrated that constituents of the glycocalyx are involved in vascular homeostasis, such as maintaining the vascular permeability barrier, regulating the release of nitric oxide and harboring a wide array of enzymes that might contribute to its vasculoprotective effect (14). There are however limited knowledge on the mechanisms by which the glycocalyx components are acting to protect the endothelium. Several studies aimed at examining the structure of glycocalyx, its response to different flow conditions and pathological stimuli as well as its effect on cell adherence and migration have been performed (14-17,19). There is evidence indicating that the glycocalyx has an important role as a transducer of mechanical forces to the intracellular cytoskeleton in the initiation of intracellular signaling (14-16). There is also evidence that suggests an effect of glycocalyx on cellular permeability (20-24) as evidenced by the release of bioactive molecules regulating vascular hoemostasis from the endothelial cells. Proteoglycans like HS and CS released from EC are known inhibitors of platelet aggregation. The efficiency of this important function is depending on the size

and quantity of the released proteoglycans, which in turn have been shown to be regulated by flow shear stress (25).

Previous efforts to describe the effects of flow stress on the morphology of the glycocalyx layer have predominantly examined the glycocalyx layer by staining after fixation of in vitro or in vivo endothelial cells. By this approach, it has been shown in bovine aortic endothelial cells that the morphology of the glycocalyx is flow dependent such that the thickness of the detectable glycocalyx layer increase with 74% when the laminar shear stress is increased from 1 Pa to 3 Pa (20). Furthermore, endothelial cells isolated from regions of different levels of laminar shear stress in mice aorta showed a higher presence of glycocalyx components in the regions of higher shear stress. (26). Flow shear stress effect the morphology of the glycocalyx layer by stimulating the glycosaminoglycan synthesis in PAEC in vitro after 24 h of shear stress, resulting in an increase in the glycocalyx layer thickness (27).

Transport and permeability

The accumulation of lipoproteins debris, plaques, in the arterial wall is one of the first stages in atherosclerosis. Atherosclerotic plaques consist of intracellular and extracellular lipids, smooth muscle cells, connective tissue, and glycosaminoglycans. Low density lipoprotein (LDL) is the most abundant lipoprotein in plasma and the flux on LDL through the endothelium depends on the plasma concentration as well as the permeability. The correlation between enhanced endothelial permeability to macromolecules and the localization of atherosclerotic plaques is well established.

Macromolecules can cross the endothelium either by transport through the cells, a process that requires endo and exocytosis, or by passing through leaky junctions. The latter have been associated with cell proliferation and apoptosis both processes affected by shear stress. The process of pinocytosis (fluid phase endocytosis) has been studied by Davies *et al.* 1999 (24) and they have found that continuous exposure to steady laminar flow creating a shear stress of 1-15 dynes/cm2 stimulated the pinocytotic rate. A sudden removal also resulted in a transient increase in pinocytotic rate. Other studies of shear stress dependence of the uptake of macromolecules include Ueda *et al.* 2004 (20) who report a decreased uptake of albumin with increased shear and Sprague *et al.* 1987 (23) who investigate the uptake of radioactively labeled LDLs and find that the internalization is greater under high shear (30 dyn/cm2) than under low shear(<1 dyn/cm2). Ueda *et al.* 2004 (20) show that the uptake of fluorescently labeled albumin decreases with increased shear stress Adamson 1990 (28) and Huxley and Williams 2000 (22) have investigated the role of glycocalyx on the permeability by enzymatic removal of glycocalyx structures suggesting that the presence of glycosaminoglycans on the cell surface is an important factor for the permeability of the endothelium. Cell surface heparan sulfate proteoglycan (glypican) facilitate uptake of growth-promoting polyamines as have been shown by Fransson *et al.* 2002 (47).

Several mechanisms have been suggested to explain the detrimental effect from low shear stress on vascular disease in terms of glycocalyx properties. The most prevalent view is that the glycocalyx layer is physically shielding the EC surfaces from attachement and uptake thereby its reduced thickness under low shear would explain the higher rate of onset of disease in these regions (14,20,29). We have explored the hypothesis that the glycocalyx layer is also shielding the endothelial cells by preventing active endocytosis of potentially hazardous bioactive molecules. The glycocalyx structures are known to largely collapse by the fixation procedure (Ueda *et al*. 2004 (20)). In this study, the heparan sulfate component of the glycocalyx layer of both live porcine aortic endothelial cells (PAEC) and fixed PAEC is stained with fluorescence labeled Lycopersicon Esculentum Lectin as well as antibodies. The cellular permeability is studied using Lycopersicon Esculentum Lectin and Low Density Lipoprotein (LDL) isolated from human plasma. In order to examine the cellular response to shear stress, a precise rectangular flow chamber with pressure driven flow is used where cells can be grown exposed to a well defined constant shear stress for up to 48 hours. The flow in the channel is steady laminar Poiseuille flow (stationary flow of incompressible uniform viscous liquid, so called Newtonian fluid, through a cylindrical tube with the constant circular cross-section) entering and exiting from one side to the other through circular tubes. The flow at the inlet and outlet section of the channel is three-dimensional. However, near the center of the channel, where the cells are observed, the flow is effectively a two-dimensional Poiseuille flow. Since the height of the channel is much smaller than the length and the width (Fig. 4), the velocity profile away from the walls can be considered as laminar parallel Poiseuille flow. In vitro experiments comparing the glycocalyx layer of live PAEC under flow shear stress, to cells under static conditions, show significant differences in heparan sulfate staining in live cells. Interestingly, penetration of the lectins (and probably HSPG) as well as LDL into the cytoplasm of the cells seems to be lower in the flow exposed cells indicating that this endocytosis process might be shear dependent. To test the hypothesis that endocytosis is responsible for macromolecule uptake the incubation temperature was lowered to 4 ºC and this treatment largely reduced the amount of lectins in the cytoplasm. The cells were also treated with PAO, a pharmacological endocytosis inhibitor that has been shown to inhibit receptor mediated endocytosis (but not pinocytosis) in endothelial cells (30) To elucidate whether the increased amount of HSPGs on the surface of the shear stress exposed cells are directly responsible for inhibition of endocytosis the cells were treated with HS as well as Heparinase III to artificially increase or decrease the glycocalyx thickness. Addition or removal of heparan sulfate did not have a direct effect on the uptake of macromolecules.

It is well established that HSPGs play a role in the LDL uptake in endothelial cells. The prevalent model is that the LDL is transferred to the LDL receptor after binding to HSPG but it is also suggested that a small portion of HSPG bound LDL is internalized directly (31). The binding and internalization of LDL was here investigated by incubation of the cells with fluorescently labeled LDL after shear stress exposure. Both LDL immobilized at the surface and internalized LDL is largely reduced by shear stress, and co localization of LDL and lectins provide evidence that these molecules are endocytosed as a complex.

MATERIALS AND METHODS

Design of the flow chamber

A perfusion system was designed (Fig. 6) where the cells could be grown exposed to a defined amount of shear stress for extended periods of time. Important features of the system are accurate temperature control, an atmosphere of 5% CO_2 in order to maintain physiological pH in the medium, direct visualization of the cells while in the chamber and sterility. I found that FCS2 closed system from Bioptechs inc. was able to solve the temperature problem as well as the visualization of the cells. With a peristaltic pump from Harvard Apparatus I could create a high enough shear to simulate aortic conditions and the flow profile was determined accurately with Micron-Resolution Particle Image Velocimetry, by Hong Sun, PhD student. (Data not shown) The dimensions of the chamber used are specified in figure 5 and equation 1 was used to calculate the shear stress (table. 1) assuming that the viscosity of the medium is close to water.

$$Shear\ stress = \frac{3}{2} \cdot \mu \cdot \frac{v}{w \cdot \left(\frac{d}{2}\right)^2} \quad \text{(eq.1)}$$

Where μ = viscosity, v =flow velocity, w = width of chamber and d = depth of chamber. The following parameter values were used to calculate the shear:

$\mu = 995.9\ {}^{kg}/_{m \cdot s}$

$d = 0.25 \cdot 10^{-3}$ m

$w = 14.5 \cdot 10^{-3}$ m

Table 1. Flow rate and corresponding calculated shear stress

v = [ml/min]	[dyne/cm^2]
9.00	9.87
9.50	10.42
10.00	10.97
10.50	11.52
11.00	12.07

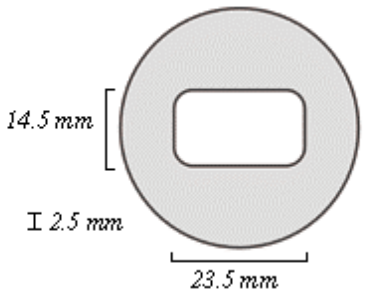


Figure 5. Dimensions of the flow chamber: Length: 23.5 mm, width: 14.5 mm and height: 2.5 mm. The rubbergasquet in the image is placed between two glass slides to create the chamber.

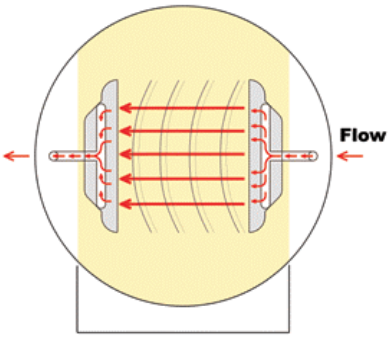


Figure 6. Schematic image of the flow profile in the perfusion chamber. The flow is approximately a two-dimensional Poiseuille flow near the centre of the chamber.

A proper atmosphere was created by connecting a gas inlet to the medium container allowing a slow stream of a 5% CO_2 gas mix bubble through the medium. The flow was stabilized by introducing a second medium container, hermetically sealed to create a vacuum, in the loop. Filling this container partly creates an air cushion and the surface separating the air from the medium works to damp the pulse created by the peristaltic pump. Once assembled the system has no exchange with the environment making it possible to maintain sterility throughout the entire experiment. A syringe connected to a two-way valve allow for injections of substances into the system without breaking the sterility.

To be able to monitor the cells without removing them from the flow the perfusion chamber was mounted on a Leica inverted light microscope and images were taken using a Hamamatsu camera and Simple PCI imaging software.

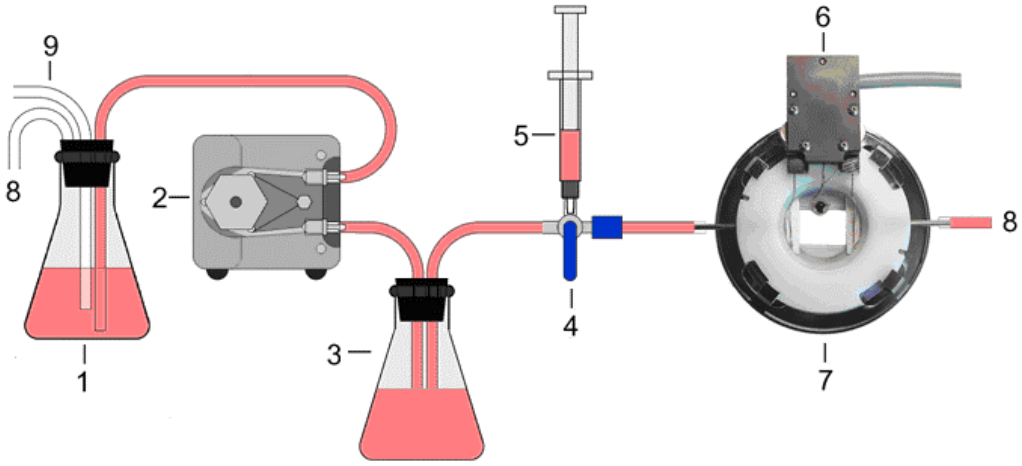


Figure 7. Schematic image of the perfusion loop 1. Medium container 2. Peristaltic pump 3. Puls damper 4. Two-way valve 5. Syringe 6. Temperature control devise 7. Perfusion chamber 8. Connecting tube 9. Gas inlet.

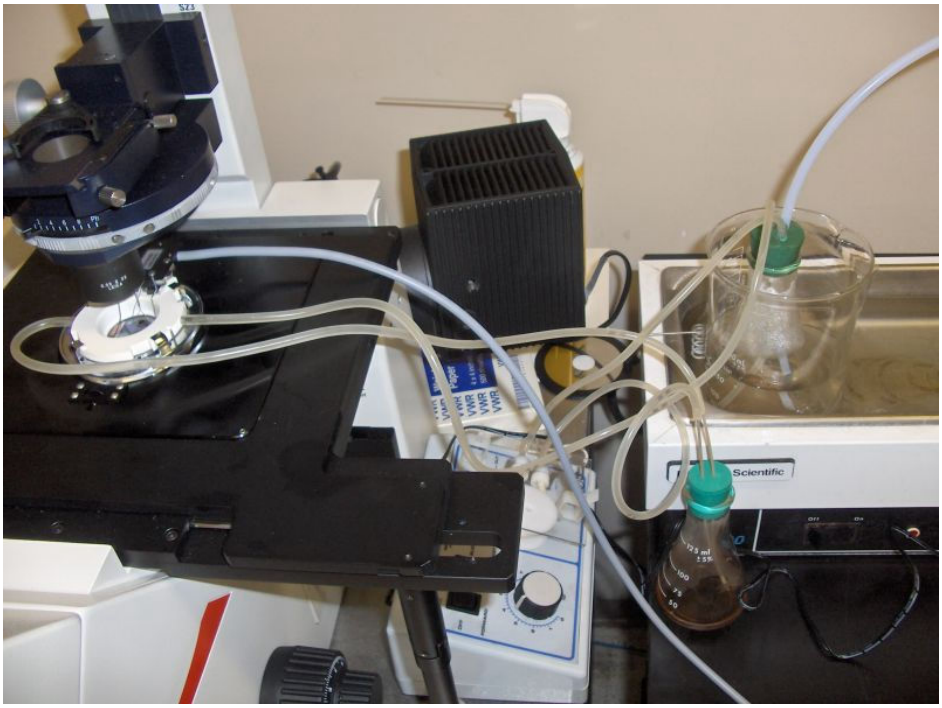


Figure 8. The flow chamber in action!

Cell culture and shear stress experiments

Porcine aortic endothelial cells (PAEC) were purchased from Cell Systems Inc. All cells used were from passage 1-5 and were grown to confluency in complex medium (EGM-MV, Cambrex). For the shear stress experiments the medium was changed to EMDM with HEPES containing 10% FBS and 2 mM L-glutamin before the cells were introduced in the flow chamber. Cells were grown on fibronectin coated cover slides prepared fresh for each experiment. Confluent cells were transferred to the perfusion chamber where they were exposed to steady laminar shear stress of 11 dynes/cm^2 for 24 h or 48 h keeping a constant temperature of 37ºC and a 5% concentration of CO_2 in the atmosphere. Control samples were grown in no-flow conditions in the same medium as used for the perfusion chamber. Some of the cell samples were fixed before staining. The fixation of the cells was performed with 3.7% paraformaldehyde that was added to the monolayer immediately after removal from the flow chamber. Cells were fixed for 15 min and washed 3 times 10 min in DPBS containing 2mM Glycine.

Histochemistry and confocal microscopy

The cells were stained for heparan sulfate using 10 µg/ml Texas-Red or FITC conjugated Lectins (*Lycopersicon Esculentum*, vector laboratories) in DPBS. Celltracker Orange, 3 µM (Cambrex) or Hoechst, 0.5 µg/ml (Cambrex) were used as reference stains. Staining was performed in stationary condition and samples were incubated for 30-90 min in 37ºC or 4ºC. For the immunofluorescense experiments monoclonal antibody (MAB2040, Chemicon) against heparan sulfate was used together with FITC conjugated secondary

antibody (AP124, Chemicon). Samples were incubated with DPBS containing 1% BSA and 5 mM $CaCl_2$ for 30 min before antibody treatment to block unspecific binding. The stained cells were visualized using a LSM 510 confocal scanning microscope. By varying the distance between the pinhole and the specimen, z-series of images were created making it possible to dissect through the cell monolayer. Two different channels were used for two different wavelengths preventing "bleeding" of the flourocromes. A minimum of three image stacks were collected from each sample and for the shear stress exposed samples the locations of the images were restricted to the center of the chamber. The presented images were created using LSM Image browser.

Heparinase/Heparan Sulfate treatment

Following shear stress exposure cells were treated with Heparinase III (Sigma) by incubation with enzyme (1 U/ml) in 20 mM Tris-HCl buffer (pH 7.5, 4 mM CaCl2, 0.1 mg/ml BSA) for 45 min. Control cells were kept stationary and treated identically. The cells were then stained for heparan sulfate using Texas Red labeled lectins as described above. Stationary cells were incubated with 1 mg/ml heparan sulfate (Sigma, H7640) in serum free medium (DMEM, Cambrex) for 90 min before lectin staining. A control sample were incubated with DMEM only and stained with lectin identically. Stationary grown cells were also exposed to HSPG (Sigma H4777) that had been incubated with lectins for 60 min before addition to the cell cuture. The solution was prepared with 2 μg/ml lectin and 5 μg/ml HSPG in DMEM. Assuming a molecular weight of >400 kDa for the HSPG and 100 kDa for the lectins and an average number of 4-6 GAG chains/HSPG most of the lectins should be in complex with the HSPG after 60 min of incubation. The cells were incubated with the HSPG/lectin solution for 30 min before visualization with confocal microscopy. Control samples were incubated with lectins only.

Endocytosis inhibition

To prevent endocytosis the cells were incubated at 4°C or treated with 1 μM Phenyl Arsine Oxide (PAO) before and/or during incubation with lectins/LDL. PAO was dissolved in DMSO and than diluted to a final concentration of 1 μM in serum free medium (EMDM) or DPBS.

LDL uptake and immobilization

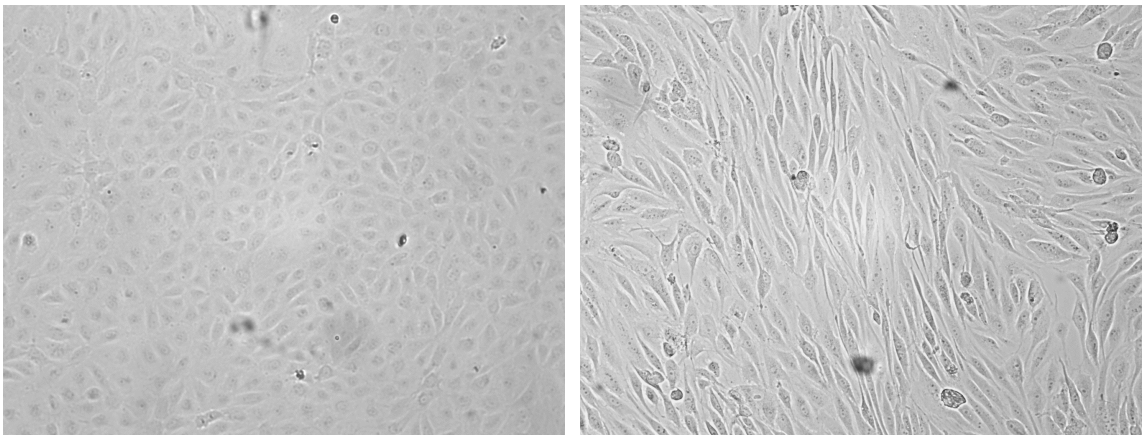
After shear stress exposure for 24 or 48 hours the cells were incubated with DiI-LDL (Invitrogen L-3482, 10 μg/ml) and FITC-LEA (Vector laboratories, 10 μg/ml) in DPBS for 60 min, 37°C 5% CO_2. The distribution of stain intracellularly as well as immobilized on the surface was examined with confocal microscopy.

RESULTS

Effect of shear stress on lectin distribution

The effect of shear stress on the glycocalyx structure was examined by exposing PAEC to flow shear stress at 11 dynes/cm2 for 24 h or for 48 h, or no flow in a static culture condition. An alignment with the flow direction could be observed in the shear stress exposed cell monolayer (Fig.9). The glycocalyx was visualized by staining of live cells with Texas-Red conjugated lectin LEA specific for heparan sulfate. Examination with confocal microscopy showed an increase in surface staining on cells exposed to laminar flow stress over time (Fig. 10). Cells exposed to shear (Fig 10 D-H) showed distinct border stains outlining the cell surfaces that were not present in the cells grown under static conditions (Fig. 10 A-C). The surface stain was more prominent after 48 hours suggesting that incorporation of heparan sulfate in the glycocalyx layer was still increasing after 24 hours of shear stress exposure. The stain appeared equally throughout the inside of the cells grown under static conditions. Cross section of the confocal images confirmed that the stain was located on the surface of the cells exposed to flow stress (Fig. 11A) and in the cells grown in static condition, the stain was located in the interior of the cells (Fig. 11B).

The staining pattern observed for the shear stress exposed cells was more prominent in the cells that had aligned with the flow direction than in the cells that were still in transition (Fig. 12).



A **B**

Figure 9. Confluent PAEC, passage 2 grown to confluency in static conditions with in EGM-MV (Cambrex) then transferred to EMDM (Cambrex) supplemented with 10% FBS and 2 mM L-glutamine. A: Cells grown in static conditions for 48 hours. B: Cells grown in a flow chamber exposed to a shear stress of 11 dynes/cm^2 for 48 hours, flow direction vertically in the image.

Tracking of Heparan Sulfate with FITC conjugated antibodies

To confirm the difference in abundance of heparan sulfate between shear stress exposed cells and control cells, samples were fixed and counter-stained with FITC conjugated antibodies towards human heparan sulfate and Cell Tracker Orange. The cells that were grown under flow shear stress show labeling on the cell surface (Fig. 13A,C). Less staining was observed on the surface of the cells grown under static condition (Fig. 13B,D). Cross sections of the confocal image stacks confirmed the observations (Fig, 13E).

Endocytosis inhibition

To further characterize the uptake of FITC/Texas red conjugated lectins into the cells, confluent cells from the two experimental growth conditions were stained with lectins and counterstained with CellTracker Orange at +4 °C. The live cells from the static condition for 48 h showed staining predominantly on the inside of the cells and limited staining on the outside at 37 °C (Fig. 14A), as was also previously shown in Fig. 1A- C. At +4 °C, there was a clear staining of the outside of the cells and no apparent staining on the inside (Fig. 14C). Cells grown under flow stress showed staining on the outside and limited staining on the inside at 37 °C (Fig. 14B). Experiments with Texas-Red conjugated LEA only confirmed the results as shown in Fig. 15. The cells were also treated with PAO, a pharmacological endocytosis inhibitor that has been shown to inhibit receptor mediated endocytosis (but not pinocytosis) in endothelial cells (30), prior to staining with Texas-Red conjugated lectins. This treatment considerably reduced the internalization of stain (Fig 16). These results support the hypothesis that uptake of stain is due to receptor mediated endocytosis.

Effect of Heparan Sulfate/Heparinase III treatment

To investigate the role of heparan sulfate as a possible inhibitor of lectin endocytosis the cells were treated with Heparinase III following shear stress exposure. Stationary cells were treated identically as a control. The experiment showed no increase of endocytosis after Heparinase III (1 U/ml) treatment in neither the shear stress exposed cells nor the control (Fig. 17). However there was a reduction of stain in the shear stress exposed cells (Fig. 17B) compared to those not treated with Heparinase III (Fig. 10G-I). Cells grown under static conditions did not show any difference in heparan sulfate staining after HepIII treatment (Fig 17A, Fig 10A-C). When stationary grown cells (Fig 18A) were treated with heparan sulfate (1 mg/ml) prior to staining there was no decrease in endocytosis compared to the cells receiving no treatment (Fig 18B).

To confirm the attachment of HS to the cell surface the cells were soaked in heparan sulfate and stained with FITC lectins and CellTracker Orange. Endocytosis was inhibited with PAO. The HS soaked cells (Fig20B) have a conciderable larger amount of FITC lectins attached to the surface compared to the control (Fig20A). The amount of bound HS was determined by examining the realtive fluorescence in the two images. Since no

absolute quantification of the number of HS molecules has been done, this result should only be considered as a positive indication of the effectivenes of the HS incorporation.

HSPG uptake in static cultures

It is possible that the decrease of surface lectins on the surface of stationary grown cells is due to an increased turnover of HSPG in absence of flow. To test the ability of the cell to internalize HSPG in complex with lectins stationary grown cells were exposed to a mixture of lectins and HSPG before visualization. The solution was prepare so that the number of heparan sulfate side chains acting as lectin binding sites should exceed the number of lectins and incubated for 30 min before addition to the cells. The amount of free lectin was expected to be very small after incubation with HSPG. The results are shown in Fig 21. There was no significant difference between the sample treated with a mix of HSPG and lectins (Fig 19A,C,E) and the control sample (Fig 19B,D,F) which had been treated with an identical amount of free lectins. Since the addition of HSPG does not considerably affect the cells ability to internalize the lectins it is possible to conclude that the cell does indeed take up the whole lectin-HSPG complex.

Effect of shear stress on LDL up-take and immobilization

Confocal images of PAEC incubated with DiI-LDL and FITC-LEA show a marked decrease in macromolecule uptake in the shear stress exposed cells compared to the control (Fig21). There were also considerably less LDL immobilized on the cell surface on cells exposed to shear stress. Cross sections of the image stacks confirmed the location of the stains (Fig22). Examination with multi channel confocal microscope allow for detection of co localization of the stains (yellow). A large proportion of the internalized LDL appeared to co localize with the lectins suggesting that LDL and heparan sulfate were endocytosed as a complex. Cells were also incubated with DPBS containing LDL and lectin together with PAO (Fig. 23B). This treatment largely reduced the internalization of both LDL and lectins compared to the control (Fig. 23A), confirming that receptor mediated endocytotsis was responsible for LDL uptake.

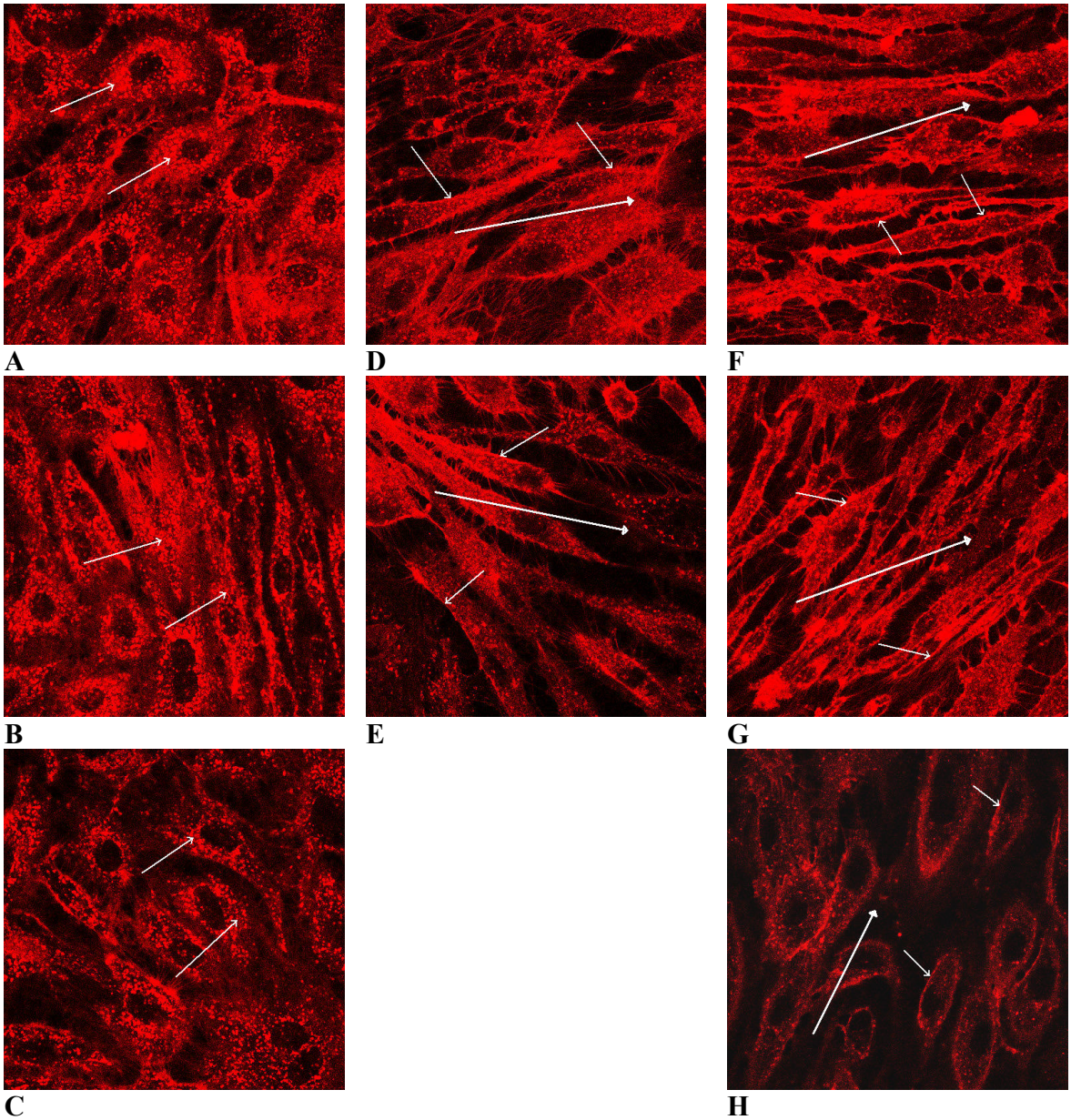
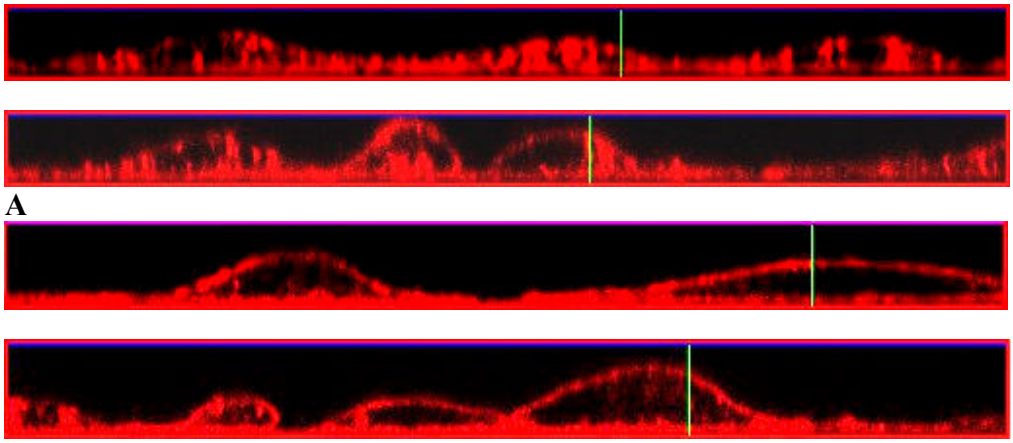


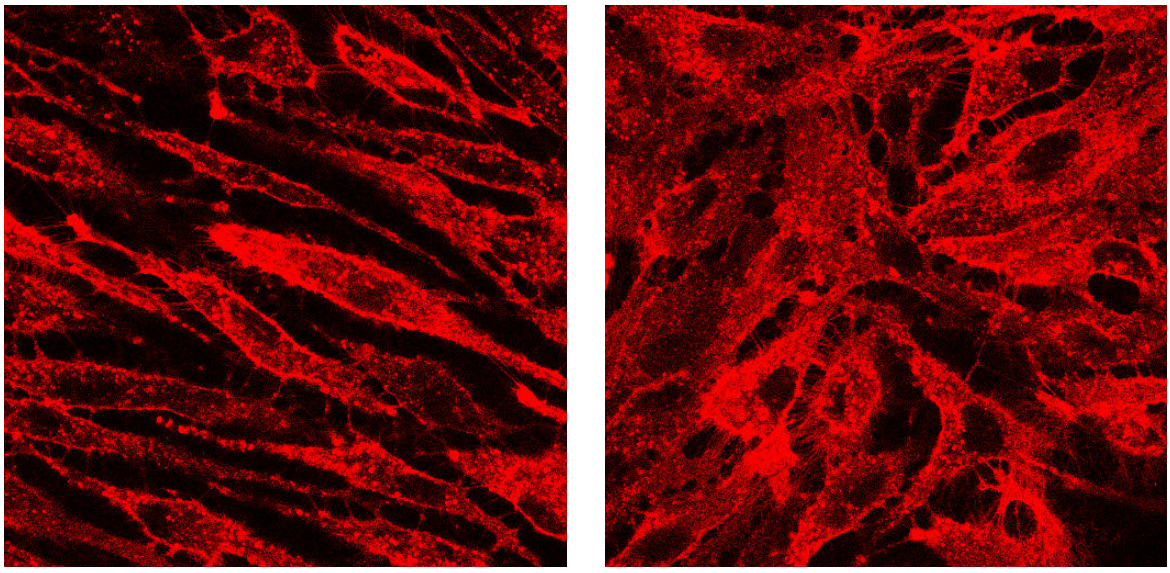
Figure 12. Confocal images of PAEC grown on fibronectin coated glass slides in DMEM supplemented with 10% FBS and 2 mM L-Glutamine and stained alive for heparan sulfate (Texas-Red conjugated *Lycopersicon Esculentum* Lectin, 10 µg/ml) after exposure to shear stress for 0h, 24h or 48h. A-C: Cells grown in no shear conditions 24 hours. D-F: Cells grown for 24 h exposed to laminar shear stress of 11 dynes/cm^2. G-I: Cells grown for 48 h exposed to laminar shear stress of 11 dynes/cm^2. The images are cross sections of the cell monolayer z=0.5-2 µm with z=0 at the glass surface. Large arrows indicate approximate flow directions. Small arrows indicate the distribution of the stain, predominantly located in the interior of the cells not exposed to shear stress compared to the cells exposed to shear stress were it was most abundant at the surface.



A

B

Figure 13. Cross sections from the side of the stack of images created with confocal microscopy of A: Cell monolayer grown in static conditions. B: Cell monolayer exposed to laminar flow, 11 dynes/cm^2 , for 48 hours. The flow exposed cells show a homogenous staining of the surface, while the stationary control has considerable staining throughout the whole cell.



A **B**

Figure 14. Confocal images of cells that has been exposed to laminar flow of 11 dynes/cm^2 for 48 hours. Image A show cells that has aligned with the flow and B show cells that are still in transition. The images are cross sections of the cell monolayer z=0.5-2 µm with z=0 at the glass surface. The staining pattern observed for cells exposed to shear stress is not as obvious in the cells that have not yet aligned with the flow direction.

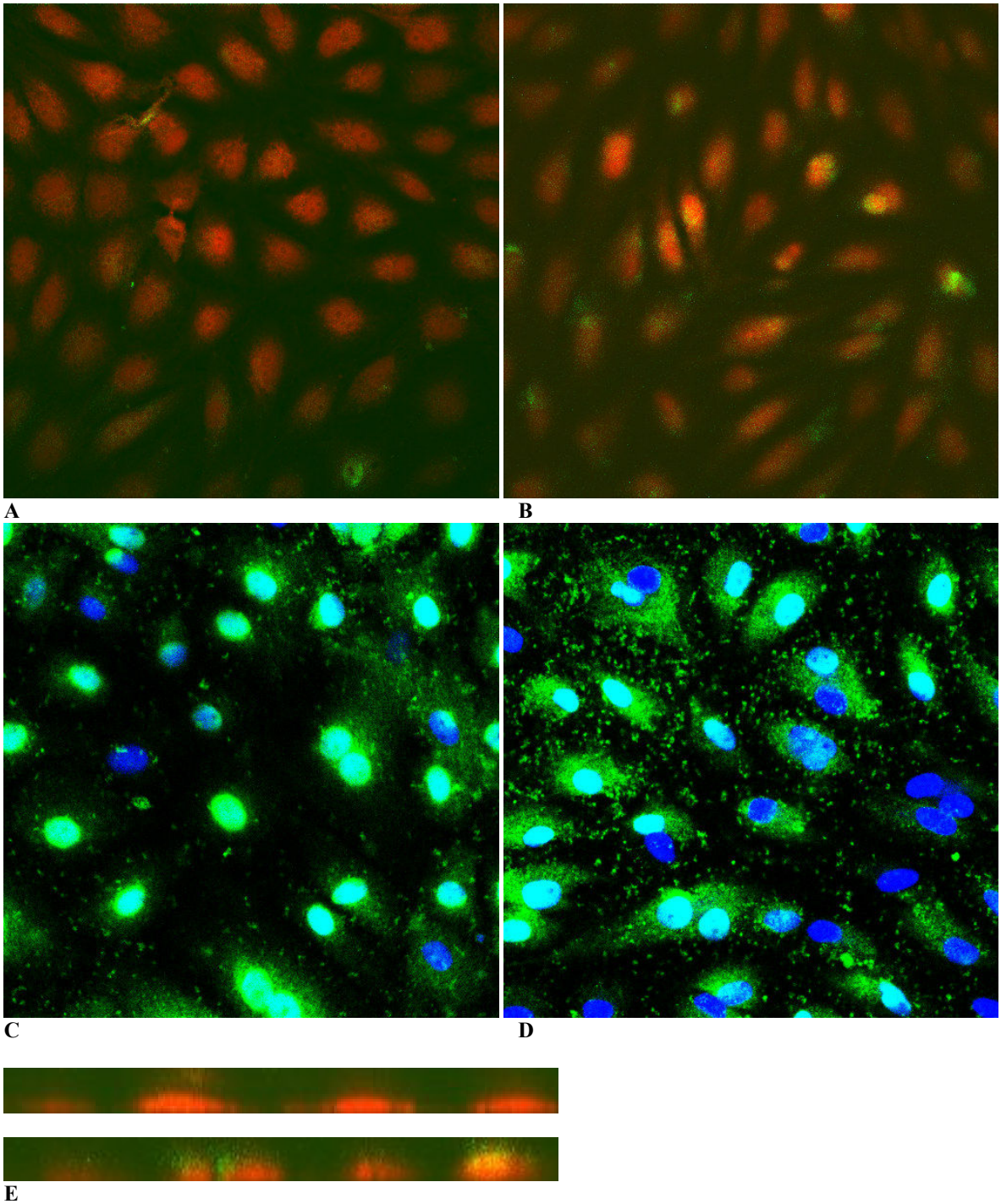
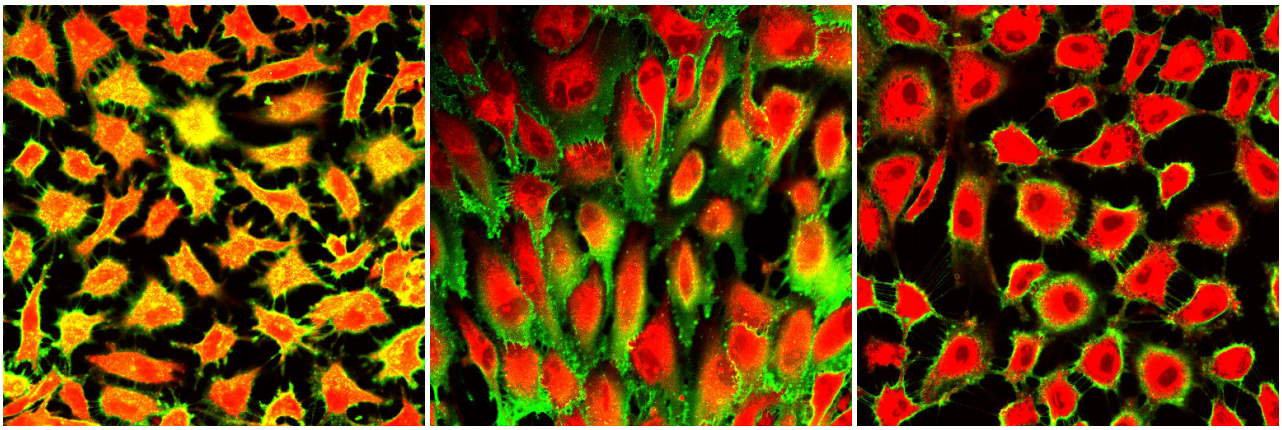
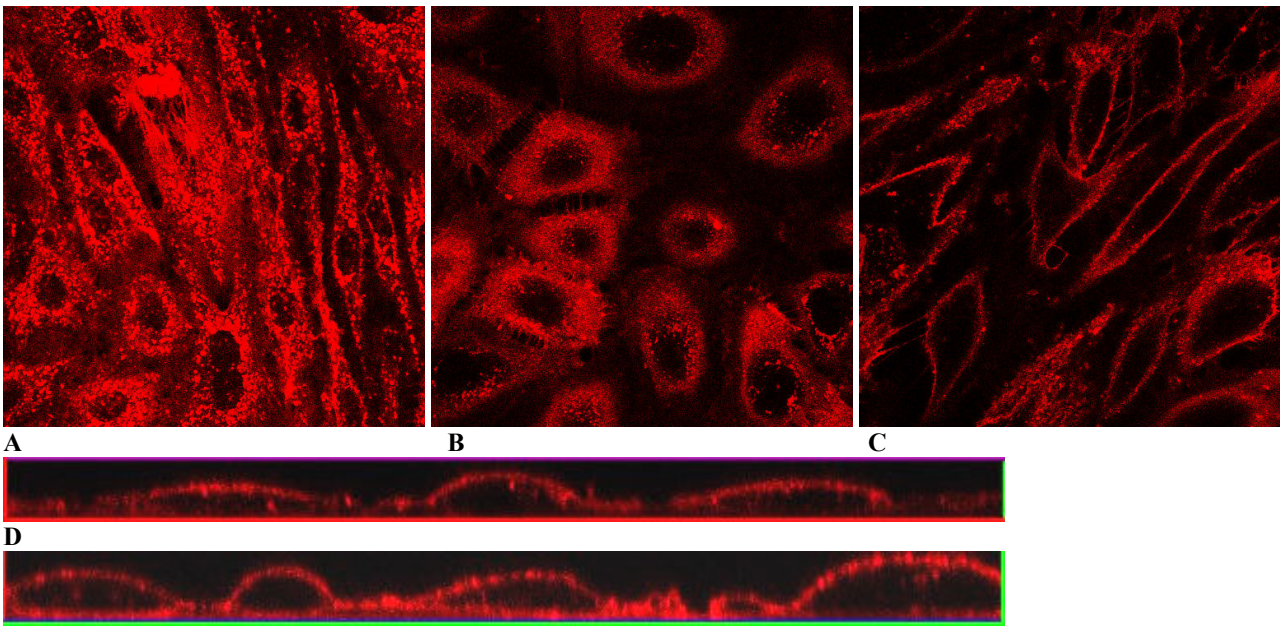


Figure 15. Confluent PAEC, passage 2 grown to confluency in static conditions with in EGM-MV (Cambrex) than transferred to EMDM (Cambrex) supplemented with 10% FBS and 2 mM L-glutamine. The images are cross sections of the cell monolayer z=0.5-2 µm with z=0 at the glass surface. A,C: Cells kept in constant condition for 24 h. B,D: Cells exposed to laminar shear stress of 11 dynes/cm^2 for 24 h. Cells are fixed for 15 min in 3.7 % paraformaldehyde and stained with FITC conjugated antibodies against heparan sulfate (Chemicon), green, and CellTracker Orange (Cambrex), red (A,B) or Hoechst (Cambrex), blue (C,D). E: Cross sections of the image stacks from the side show the location of the antibodies (green) in relation to the cytoplasm (red).



A B C

Figur 16. Confocal images of PAEC grown on fibronectin coated glass slides in DMEM supplemented with 10% FBS and 2 mM L-Glutamine and stained alive for heparan sulfate (FITC conjugated *Lycopersicon Esculentum* Lectin, 10 µg/ml), green. CellTracher Orange (Cambrex) is used as a reference stain, red. The cells were incubated with the stain for 60 min in 37 ºC or 4 ºC after shear stress exposure. Cells exposed to shear stress are grown for 48 h with a laminar shear stress of 11 dynes/cm^2. Controls are grown in stationary conditions. A: Control cells incubated at 37 ºC. B: Cells exposed to shear stress incubated at 4 ºC. C: Control cells incubated at 4 ºC. All images are cross sections of the cell monolayer z=0.5-2 µm with z=0 at the glass surface. The statically grown cells incubated at 37 ºC shows a larger proportion of internalized stain (yellow) than do the cells exposed to shear stress or the cell incubated at 4 ºC.

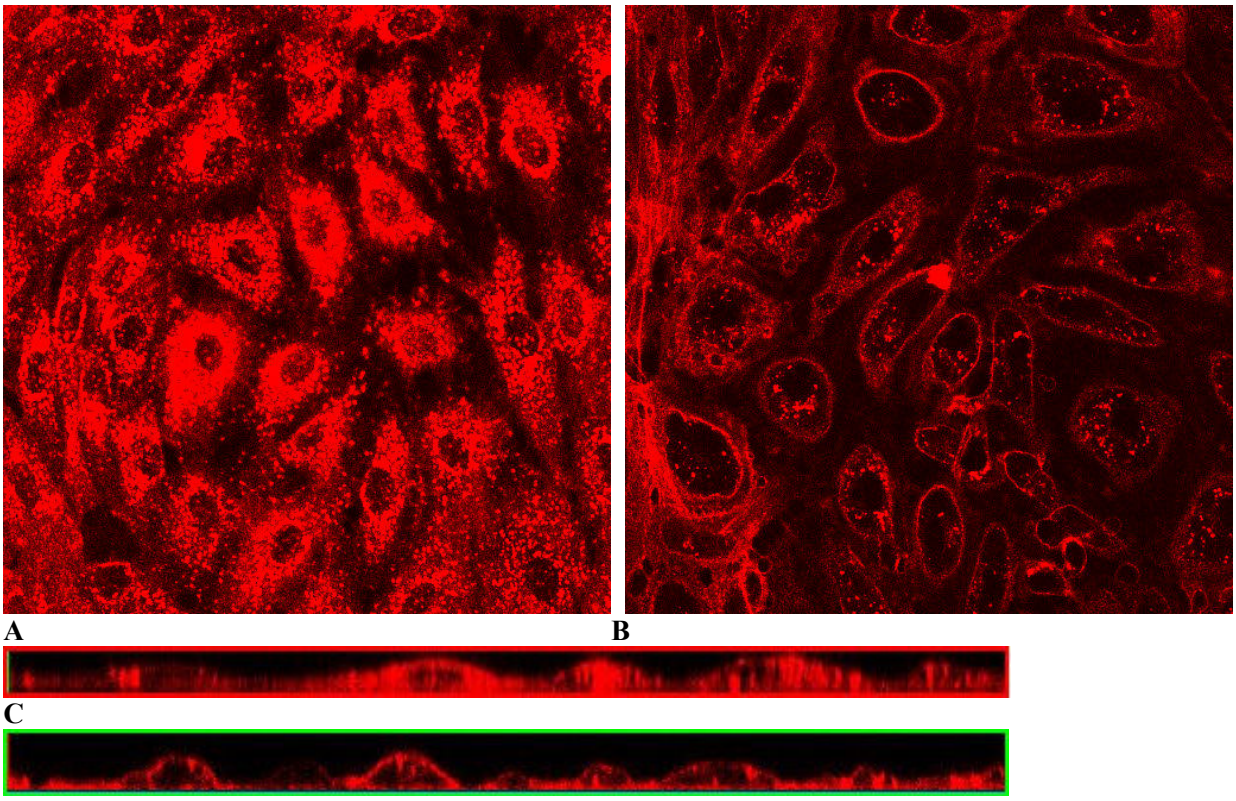


A B C

D

E

Figure 17. Confocal images of PAEC grown on fibronectin coated glass slides in DMEM supplemented with 10% FBS and 2 mM L-Glutamine and stained alive for heparan sulfate (Texas-Red conjugated *Lycopersicon Esculentum* Lectin, 10 µg/ml). A: Cells grown in no shear conditions 24 hours and incubated with Texas-Red conjugated lectins for 60 min 37ºC. B: Cells grown in no shear conditions 24 hours and incubated with Texas-Red conjugated lectins for 60 min 4ºC. C: Cells grown for 24 h exposed to laminar shear stress of 11 dynes/cm^2 and incubated with Texas-Red conjugated lectins for 60 min in 4ºC. Images are cross sections of the cell monolayer z=0.5-2 µm with z=0 at the glass surface. D: Cross section of image stack (B) of static control from the side. E: Cross section of image stack (C) of flow exposed cells from the side.

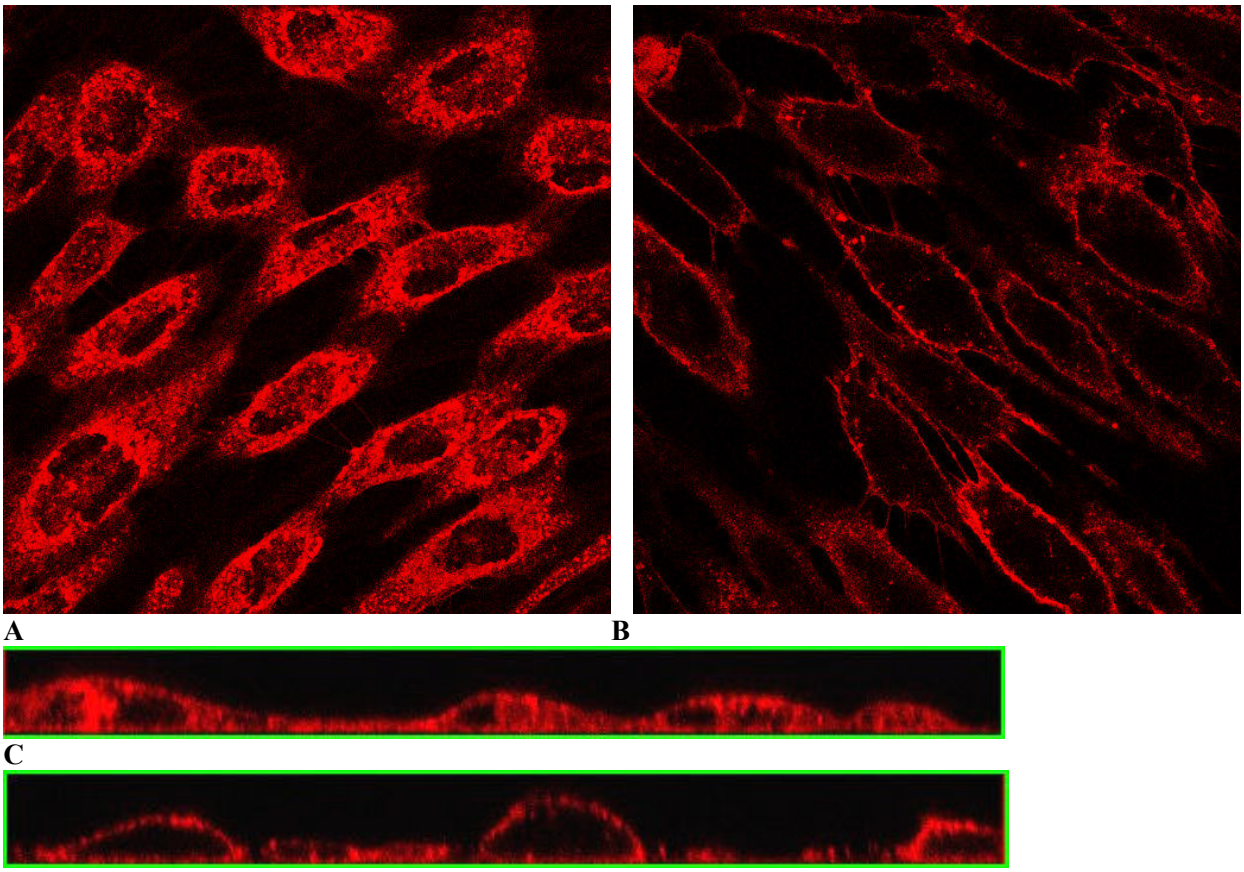


A **B**

C

D

Figure 18. Confocal images of confluent PAEC grown to confluence and stained alive for heparan sulfate (Texas-Red conjugated *Lycopersicon Esculentum* Lectin, 10 µg/ml). A: Cells grown in static conditions. 45 min prior to staining medium was changed to EMDM (serum free medium). B: Cells grown in static conditions. 45 min prior to staining medium was changed to EMDM with 10 µM Phenyl Arsine Oxide to prevent endocytosis. PAO was also included in the staining solution. Images are cross sections of the cell monolayer z=0.5-2 µm with z=0 at the glass surface. D: Cross section of image stack (B) of control from the side. E: Cross section of image stack (C) of PAO treated cells from the side.



A **B**

C

D

Figure 19. Confocal images of confluent PAEC grown to confluence and treated with Heparinase III (Sigma, 1 U/ml) before stained alive for heparan sulfate (Texas-Red conjugated *Lycopersicon Esculentum* Lectin, 10 µg/ml). A: Cells grown in static conditions and stained with HepIII for 45 min before staining. B: Cells grown for 24 h exposed to laminar shear stress of 11 dynes/cm^2 and then treated with HepIII for 45 min before staining. Images are cross sections of the cell monolayer z=0.5-2 µm with z=0 at the glass surface. D: Cross section of image stack (B) of static control from the side. E: Cross section of image stack (C) of flow exposed cells from the side.

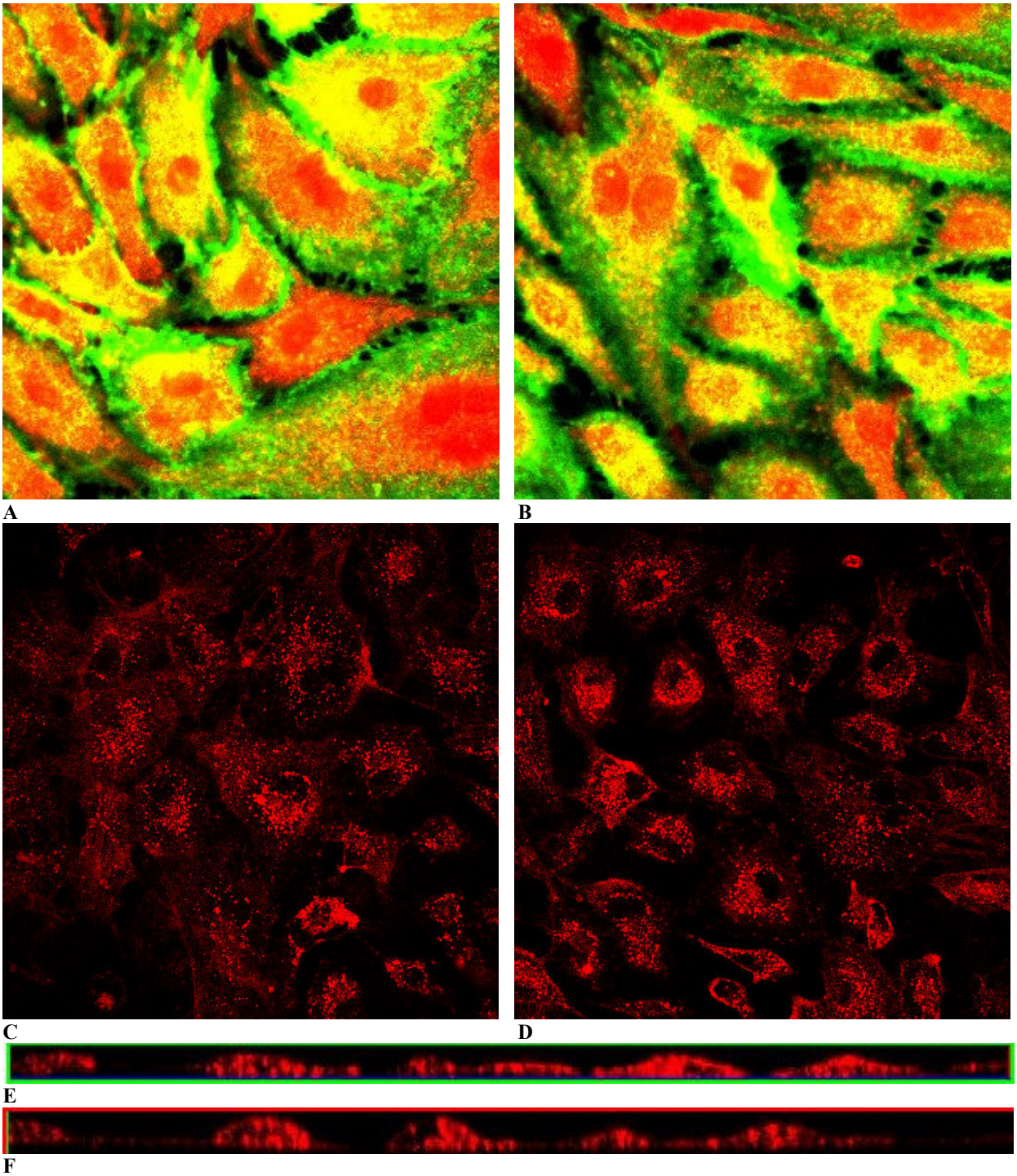
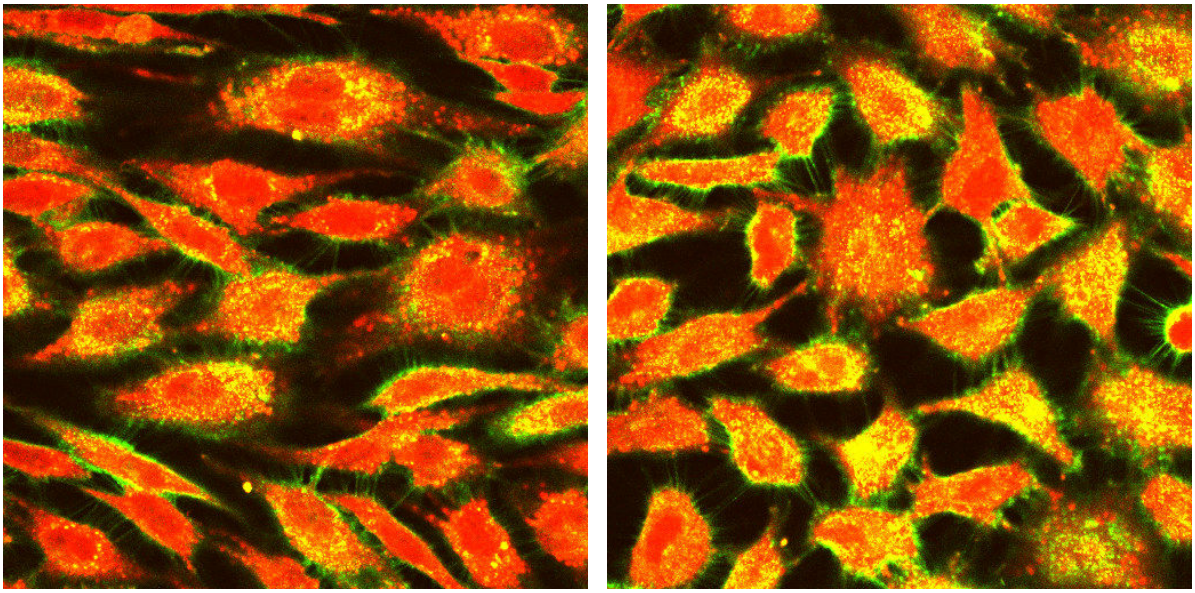
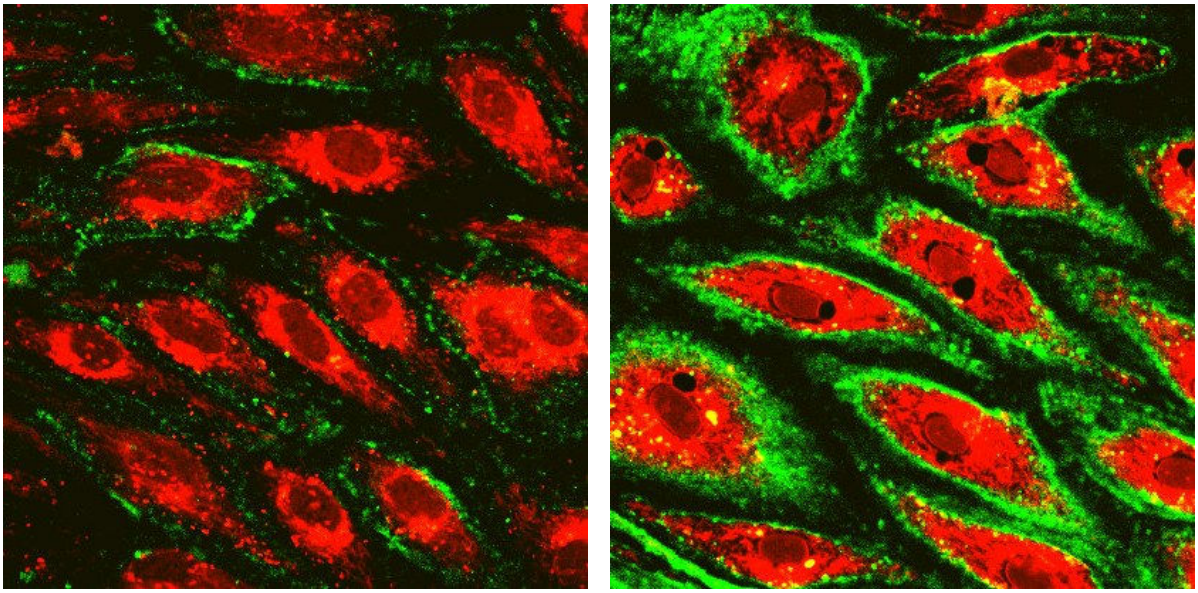


Figure 20. A: Confocal image of confluent PAEC grown to confluence and soaked with heparan sulfate (Sigma H7640, 1 mg/ml) before stained alive for heparan sulfate (FITC conjugated *Lycopersicon Esculentum* Lectin, 10 µg/ml) and cytoplasm (Celltracker Orange, Cambrex.) B: Neagtive control. C: Confocal image of confluent PAEC grown to confluence and soaked with heparan sulfate (Sigma H7640, 1 mg/ml) before stained alive for heparan sulfate (Texas-Red conjugated *Lycopersicon Esculentum* Lectin, 10 µg/ml). D: Negative control. Images are cross sections of the cell monolayer z=0.5-2 µm with z=0 at the glass surface. E: Cross section of image stack (A) of static control from the side. F: Cross section of image stack (B) of flow exposed cells from the side.



A B

Figure 21. A: Confocal images of PAEC grown to confluency and stained with lectins (*Lycopersicon Esculentum* Lectin, 2 µg/ml) incubated with HSPG (Sigma H4777, 4 µg/ml) for 30 min. B: Control cells stained with 2 µg/ml lectin only. The HSPG/lectin ratio is calculated to be large enough to allow most of the lectins to be in complex with the HSPG molecules.



A B

Figure 22. A: Confocal image of PAEC grown to confluency and stained for HS using FITC-conjugated lectins, 5 µg/ml. B: PAEC grown to confluency and soaked in HS (Sigma H7640, 1 mg/ml) before staining with lectins, 5 µg/ml. All cells are treated with PAO.

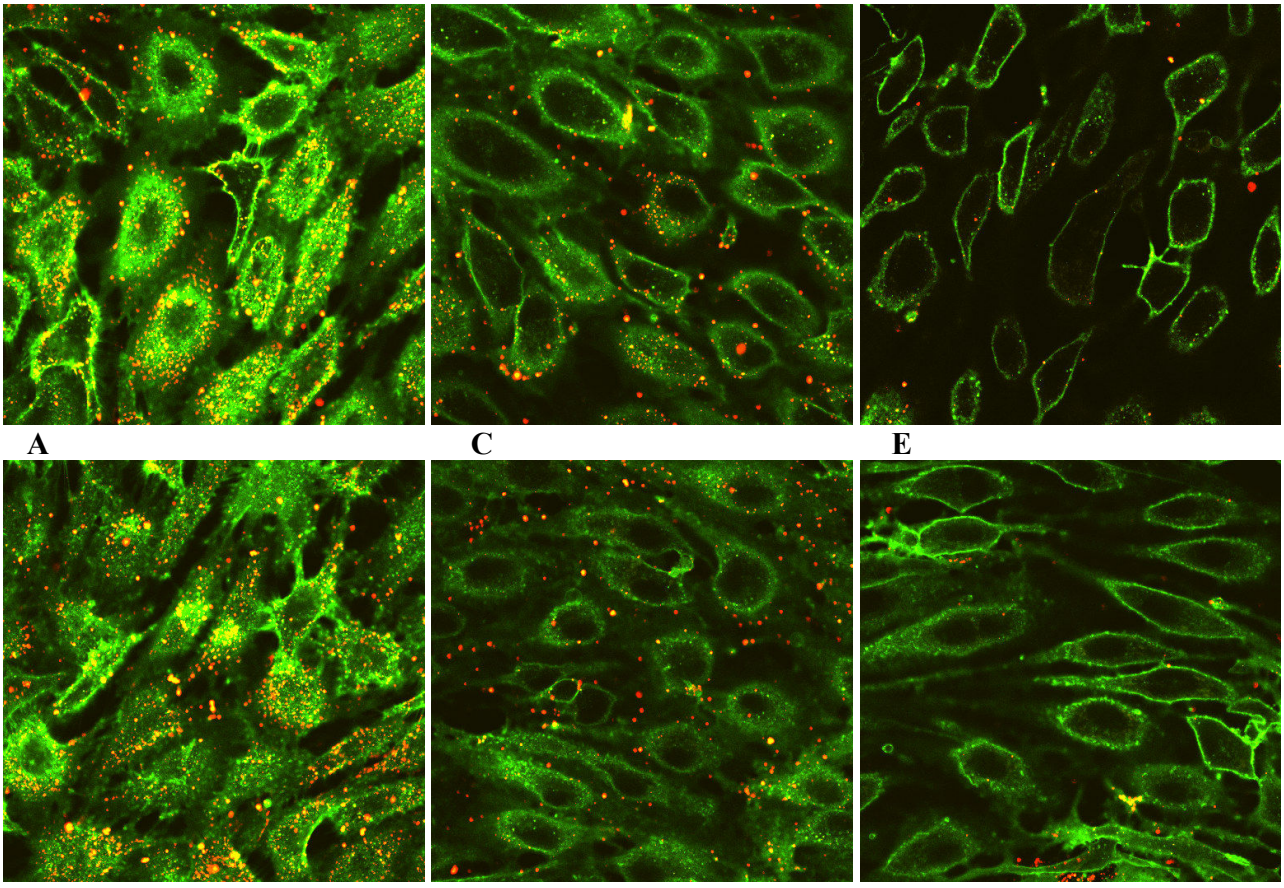
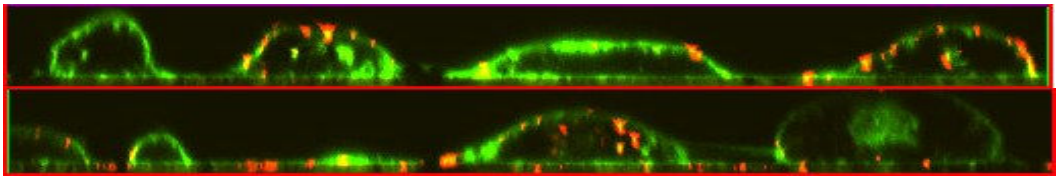
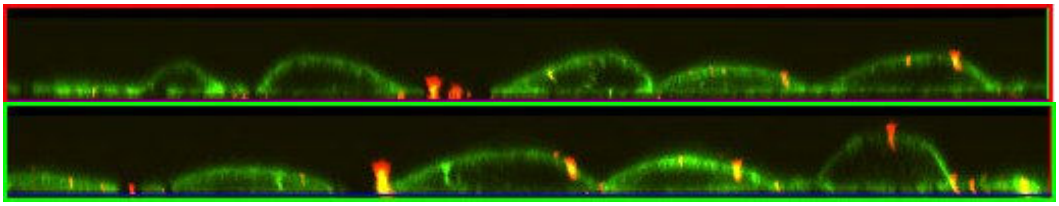


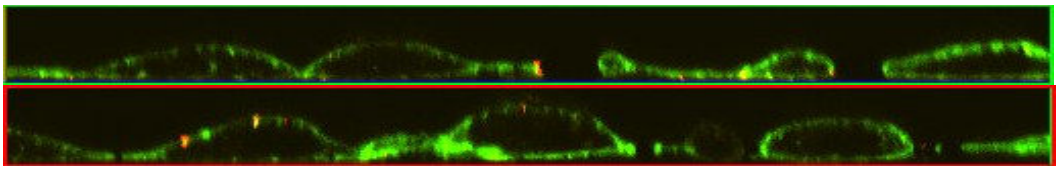
Figure 23. Confocal images of PAEC grown on fibronectin coated glass slides in DMEM supplemented with 10% FBS and 2 mM L-Glutamine exposed to shear stress of 11 dynes/cm^2 for 0,24 or 48 hours followed by incubated with heparan sulfate specific lectins (FITC conjugated *Lycopersicon Esculentum* Lectin, 10 µg/ml) green, and Low Desity Lipoprotein from human plasma, DiI complex (Invitrogen L-3482, 10 µg/ml) red. A,B: Cells grown in no shear conditions 24 hours. C,D: Cells grown for 24 h exposed to laminar shear stress of 11 dynes/cm^2. E,F: Cells grown for 48 h exposed to laminar shear stress of 11 dynes/cm^2. The images are cross sections of the cell monolayer z=0.5-2 µm with z=0 at the glass surface. Stain concentration and incubation times are similar between the experiments.



A

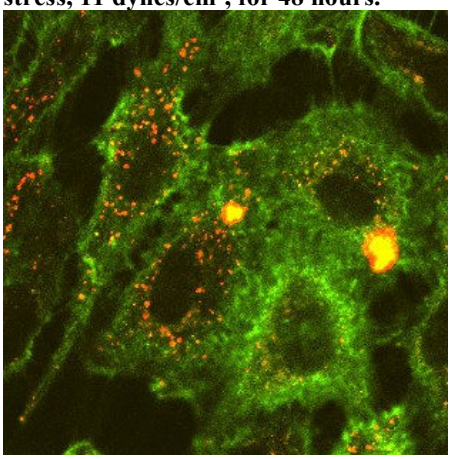


B

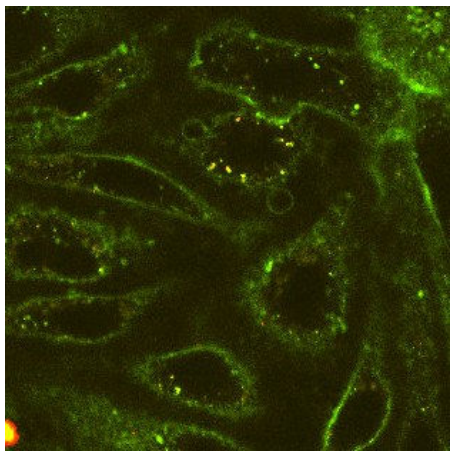


C

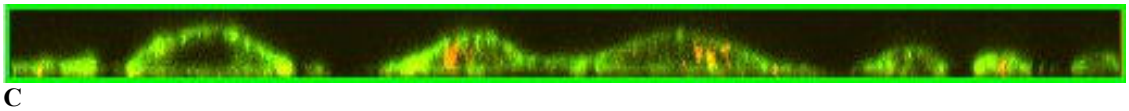
Figure 24. Cross sections of image stacks created with confocal microscopy of A: Cell monolayer in static conditions (Fig), B: Cell monolayer grown to confluency and then exposed to laminarshear stress, 11 dynes/cm^2, for 24 hours and C: Cells exposed to laminar shear stress, 11 dynes/cm^2, for 48 hours.



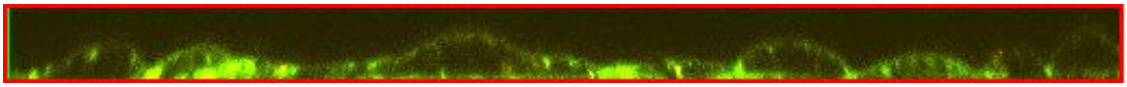
A



B



C



D

Figure 25. Confocal images of confluent PAEC incubated with A: Low Density Lipoprotein from human plasma, DiI complex (Invitrogen L-3482, 10 µg/ml) red and FITC conjugated *Lycopersicon Esculentum* Lectin (Vector Labratories, 10 µg/ml) green. B: Low Desnity Lipoprotein from human plasma, DiI complex (Invitrogen L-3482, 10 µg/ml) red, FITC conjugated *Lycopersicon Esculentum* Lectin (Vector Labratories, 10 µg/ml) green and 1µM Phenyl Arsine Oxide. The images are cross sections of the monolayer at z=0.5-2 µm with z=0 at the glass surface. D: Cross section of image stack (B) of control from the side. E: Cross section of image stack (C) of PAO treated cells from the side

DISCUSSION

In the present study, we have investigated the effect of flow stress on the thickness and function of the glycocalyx layer. We show that internalization of fluorescently labeled lectins and LDL by PAEC is greatly reduced by exposing the cells to physiological levels of steady laminar shear stress and that this internalization is not directly inhibited by heparan sulfate. Visualization of the glycocalyx of endothelial cells is challenging as common fixation procedures has been shown to collapse part of the glycocalyx structure (20). To accurately detect and measure the glycocalyx layers, we have stained the heparan sulfate within the glycocalyx layer of live cells using a lectin FITC/Texas red conjugate. Immunological staining of fixed cells using a heparan sulfate antibody showed significantly less staining of control cells compared to cells exposed to shear stress.

The extracellular presence of proteoglycans, an abundant component of the glycocalyx layer, has been shown to increase with increased levels of flow shear stress (25). It has also been shown that shear stress (15, 40 dyn/cm^2) for 24 hours significantly increased GAG synthesis, assayed by [35S]sulfate incorporation, in "medium" fraction and "trypsinated" fraction which includes GAGs derived from the cell surface and from the solubilized matrix (27) suggesting that the higher levels of heparan sulfate is partly accounted for by unbound molecules associated with the glycocalyx layer. Higher amounts of heparan sulfate, the most common GAG, a relative increase in glycocalyx thickness, under flow was demonstrated in our experimental set up, in agreement with previous studies.

The role of the glycocalyx layer in preventing the onset of vascular disease has been ascribed to the shielding properties of the matrix of glycoproteins and proteoglycans constituting the glycocalyx layer (14,20,29), preventing inflammatory substances to reach the surface of the endothelial cells. In addition, chemical properties of the glycocalyx components offer protection by direct interaction of GAG with cytokines, complement platelets and other component regulating the immune response, or release of other bioactive molecules with regulatory function (32). In early stages of atherosclerosis, it has been observed that there is an increased uptake by the endothelial cells of macromolecules and particularly of low density lipoproteins (LDLs). The uptake mechanism for LDLs has been shown to be receptor dependent and occur through endocytosis (33) and heparan sulfate proteoglycans are key players in this internalization. Interestingly, the receptor for LDLs is inhibited by sulphonated glucosaminoglycans, like heparan sulfate (34,35). Duan *et al*. 2005 (36) have found that an increase in cell surface HSPG reduced atherogenic events including lipoprotein transport. However it is also recognized by several autors (37,38,39) that the cellular uptake of LDL by endothelial cells initiates through binding to heparan sulfate which is then followed by receptor mediated endocytosis. The atherosclerotic plaques consist of accumulated intracellular and extracellular lipids, smooth muscle cells, connective tissue, and glycosaminoglycans but most of the research has been focused on the accumulation of LDL. In order to make their way to the sub endothelium these molecules have to pass through the endothelial cell layer. The intracellular junctions would not normally allow LDL molecules to pass, but cell turnover or apoptosis can result in leaky junctions, creating a possible passage.

LDL molecules can also be transported through the endothelial cells by vesicular transport and the initiating step in this process would be immobilization at the endothelial cell surface and endocytosis.

The effect of shear stress on the uptake of macromolecules has been studied by different approaches. The uptake of albumin was shown to decrease after 48 hours with increased levels of shear stress (20). This is in contradiction with data from Jo *et al.* 1991 (40) that found increased permeability of BAEC to albumin after 30 min of shear stress exposure. Similarly, when studying the internalization of radioactively labeled LDLs, this process was shown to be greater under high shear (30 dyn/cm2) than under low shear (<1 dyn/cm2) (23). The inconsistency of these data can be explained to some extent by the variation in experimental procedures. One important difference is the conditions where staining take place. In the present experiments the cells are removed from the flow chamber prior to staining, while in previous experiments showing an increased permeability with shear, the shear rate is maintained thougout the whole experiment. Also the shear rate vary beween studies as do the exposure times and concentration of macromolecules, making comparisons difficult.

The permeability of endothelial cells to low molecular weight tracers were shown to be reversible and to increase proportionally to increased shear stress (21). The rate of pinocytosis of horse raddish peroxidase relates directly to the amplitude and frequency of shear stress, such that an increase in steady shear stress increases the uptake (22,24).

Lycopersicon esculentum lectin (LEA) has been shown to penetrate the epithelial cell membrane in airways by endocytosis (41). In the present study, we show that the LEA uptake by vascular endothelial cells is eliminated by low temperature both in flow and stationary conditions (Fig. 16,17) suggesting that the LEAs are internalized by endocytosis also in the PAEC. The endocytotic mechanism was further investigated by specifically inhibiting receptor mediated endocytosis by addition of PAO, a known inhibitor of receptor mediated endocytosis (30). Stationary cells treated with the inhibitor showed significant decrease in internalized lectins, thus confirming that the lectins are processed by receptor mediated endocytosis.

Shear stress may reduce receptor mediated endocytosis in different ways. The detected higher abundance of heparan sulfate content of the surface layer under shear stress creates a more efficient physical barrier for the lectins, which is in good agreement with the idea of glycocalyx as a diffusion barrier. This hypothesis is however contradicted by the fact that no difference in lectin uptake can be detected when the HS amount is artificially increased by addition of HS to the culture. It has been shown (29,36) that it is possible to increase the glycocalyx thickness by adding HS to the medium and our results show that HS does indeed attach to the cells surface, but we fail to detect any direct effect of HS on the cellular permeability for lectins. However, it is not likely that addition of HS artificially result in the same structure as when the cell itself increases its surface content of HS. For example adding HS does not increase the fraction that is attached to the membrane through core proteins. In addition enzymatic removal of HS did not result in the predicted increase in internalized stain, but rather the opposite. However the removal

of heparan sulfate on the cells exposed to shear stress resulted in a decrease in the heparan sulfate surface layer while cells grown under static conditions did not show any difference in heparan sulfate staining. These results suggest that statically grown cells produce less heparan sulfate than do cells that has been exposed to a steady laminar shear stress for more than 24 hours, or that the turnover rate is much higher in the statically grown cells.

Some of the internalized stain might be accounted for by cellular uptake of the whole HSPG-Lectin complex as shown in figure 21. This might explain the apparent decrease in internalized stain in the Heparinase treated cells. In terms of atheroscelrosis this can be hazardous for the cell for different reasons including an increased HSPG turnover reducing the glycocalyx thickness as already observed. It might also increase the uptake of LDL since these are thought to attach to HSPG on the cell surface prior to internalization. (30,36,43). When the cells are incubated with fluorescently labeled LDL after shear stress exposure the amount of internalized LDL is indeed lower compared to the static control and colocalization of the stains indicate that some of the LDL and HSPG are endocytosed as a complex. The amount of immobilized LDL on the surface is also larger in the static control suggesting a larger abundancy of LDL-receptors on the surface of the cells grown in static conditions. The LDLs used in these experiments are not oxidized which means they are predicted to bind the lectin like LDL receptor and not the scavenger receptor that accounts for uptake of oxidized LDLs. Oxidation of LDL is an important factor in the development of atherosclerosis since oxidized LDL accumulates in the subendothelium to a larger extent than does the unoxidized form. The ox-LDL receptor is not inhibited by a high intreacellular content of LDL and thus the cells can basically eat itself to death. This is particularly true for the macrophages in the arterial wall, which, when full of internalized ox-LDL, are unable to cross the endothelium and re-enter the blood stream. OxLDL is also a chemoattractant for monocytes and is cytotoxic for endothelial cells in culture. (33) If the LDLs are abundant on the endothelial cell surface where oxaditive agents are likely released (43), this might increase the uptake of oxidized LDL and since the scavenger receptor are not thought to be inhibited by an increased concentration of LDL inside the cells, this could have large consequences for the accumulation of sub cellular LDL leading to an increased risk of atherosclerotic plaque formation.

If the uptake of macromolecules is not affected by addition or removal of HS from the cell surface, what then can explain the observed decrease in internalization of both lectins and LDL? Incubation of the cells with fluorescently stained macromolecules is performed exclusively in static conditions meaning that whatever change in the cell morphology causes the inhibition, it has to be stable enough to persist for at least a couple of hours. An increase in receptor abundance or altered receptor conformation could provide such a stable change. There has been evidence, however, that the oxLDL receptor is *up-regulated* by flow, but considering the hypothsesis that oxidation of LDL, to a large extent, occur blow the endothelial cell layer (44), the up-take of non oxidized LDL might be equally important. Another possible explanation to the decreased up-take of lectins as well as LDL in response to shear tress would be a decrease in HSPG turnover rate or that

another component of the glycocalyx also induced by shear stress is responsible for inhibition of endocytosis, one possible candidate being hyaluronic acid.

FUTURE PERSPECTIVES

During this project I have not answered many questions, but I have created a whole lot more of them for myself than I thought initially possible. How does the shear stress stimulate the endothelial cells to close its membrane to macromolecules. What is the sensor and how is the signal transduced? Can the expression of the LDL receptor on the cell surface be quantified on mRNA level? How will the cell react in response to shear if the glycocalyx is enzymatically degraded and what about varying the shear stress or the pulse to better simulate the flowing blood? By introducing a flow separation in the chamber it would be possible to create a disturbed flow profile where the cellular response to a shear gradient could be investigated. One interesting follow up to the LDL experiments would be to oxidize the LDL's to different levels before addition to the cell, alternatively to introduce the LDL already in the flow chamber.

ACKNOWLEDGEMENTS

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A special thanks to Kristiina Iisa and Jim Frederick for giving me such a warm welcome in Atlanta. Without you I would probably still be eating off the floor. A big hug to Sharron Lazarus for caring and for dealing with all the paper stuff. Thanks also to Marion Peena, who never grew tired of sending me invoice requests and the Charleses and David for computer support. Thanks to Forest biology for providing chemicals and letting me use the autoclave (when it worked) and to Bobby and Yiting for helping me out in the lab.

Finally, I want to express my deep and sincere gratitude to Gustav. You are truly no biophysicist but you are everything to me - and an excellent spell checker too.

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