HYDRODYNAMIC CONDITIONS IN A SIMPLE ROTATING MICROBIOREACTOR INDUCE CHANGES IN EXCRETION LEVEL AND GLYCOSYLATION PATTERNS OF THE Von WILLEBRAND FACTOR (vWF) IN ENDOTELLIAL CELLS

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Abstract.

There is an ongoing debate on the extent of the effect of mechanical stress (if any) on the performance of mammalian cell cultures. Here, we study the effect of low speed rotational agitation on the level of expression and the glycosylation patterns of the Von Willebrand factor in endothelial cells cultured in 6 cm Petri dishes placed at different locations on a rotating platform. In this simple system, complex and non-intuitive velocity fields arise. Flow behavior strongly varies depending on radial distance and rotational velocity value, as revealed by simple calculations of tangential velocities and CFD analysis. We found significant differences in both, excretion level and glycosylation patterns (extend and completeness of glycosylation), as a result of relatively small changes in rotational speed. Our results support the hypothesis that differences in mixing conditions may induce important changes in the secretion and assembly of glycoproteins in mammalian cell cultures.

Key words: Endothelial cells, mixing, vWF, shear stress, glycosylation, secretion.

INTRODUCTION

In this contribution, we report significant changes on the secretion of Von Willebrand Factor (vWF) and in two particular indicators of glycosylation (trimannose and sialic acid concentration) when endothelial cells are exposed to incremental rotational fields in Petri dishes. The vWF is a well-characterized plasma circulating glycoprotein produced and secreted particularly by endothelial cells (see for example Vischer 2006). vWF plays essential roles in hemostasis. It mediates platelet adhesion to the vascular wall, platelet aggregation, and serves as a plasma carrier for factor VIII, stabilizing it in the circulation. Also, it has been proposed as a marker for prognosis in primary pulmonary hypertension patients (Collados *et al.*, 1999), thrombosis and cardiovascular disease (Vischer, 2006). The over-secretion of vWF

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has been associated to the complex biochemical cascade implicated in thrombotic events (Galbucera *et al*., 1997). The role of vWF in platelet adhesion is particularly important under conditions of high shear stress.

Indeed, it is well known that certain mammalian cells (i.e. endothelial vascular cells) are particularly responsive to mechanical stresses (Dangaria and Butler 2007, Galbucera *et al*., 1997). Typical local velocity values of arterial flows promote alignment to the flow field and secretion of vasodilatant and anti-coagulant compounds (Macintire *et al*., 1998). However, when abnormal flow conditions occur in arterial flow, endothelial cells might also produce substances that promote vasoconstriction, coagulation and platelet aggregation (Sokabe *et al*., 2004). Indeed, some crucial metabolic processes related to the coagulant cascade or cardiovascular disorders are triggered or modulated by the hydrodynamics of blood in veins and arteries (Chiu *et al*., 2005).

MATERIAL AND METHODS

Isolation, cell culture and propagation of endothelial cells. Human Umbilical Vein Endothelial Cells (HUVEC) were isolated from umbilical human cordons following protocols detailed elsewhere (Rodríguez *et al*., 2002; Morga-Ramírez, 2007). Cells were recovered by incubation of the umbilical cordon vein with HSS-0.2% colagenase type II (Gibco BRL) for 15-20 minutes at of 37°C. Cells were centrifuged, washed, and re-suspended in supplemented culture media M199 [20% FBS (Gibco 12318-020), 5 U/ml heparine, 1% of antibiotics solution (penicillin and streptomycin), 10 mM HEPES, 2 mM glutamine, and 0.40 mg/ml of Endothelial Growth Factor]. Cells were seeded in culture bottles of 25 cm^2 (Corning) in 4ml of supplemented M199 culture media (previously described) and incubated at 37°C and 95% relative humidity in a 7% CO_2 and 93% air atmosphere.

Experimental system. Cells were culture in polystyrene Petri dishes (6 cm diameter) from Corning®, U.S.A. Petri dishes were placed in a rotating platform of 25 cm in diameter, at various radial distances with respect to the axes of rotation (see figure 1). Confluent cultures, normally obtained after 7 days of culture, and changing culture media every second day, were obtained in static conditions (0 RPM). Afterwards, cultures were exposed to different rotational laminar velocity fields. Here, we report results from experiments conducted at 0, 3, 5, and 8 RPM. Levels of secreted vWF after 24 hours of exposition to rotational fields were evaluated using an immunoassay technique. In addition, extent of glycosylation was evaluated by measuring the amount of trimannose incorporated to the vWF secreted at each experiment (technique described below). Level of glycosylation completeness was evaluated by measuring the amount of sialic acid incorporated to the secreted vWF in each experiment (technique described below).

Flow characterization using CFD. Computational fluid dynamics (CFD) was used to characterize flow behavior within Petri dishes rotating at different velocities. A solid model of the initial fluid volume of the Petri dish was made using the parasolids capability of Synergy, from Weber Systems Inc., Menomonee Falls, WI. The solid model was imported into the solver environment used in this work (ACUSOLVE CFD) using the acuConsole program also from ACUSIM Software Inc.

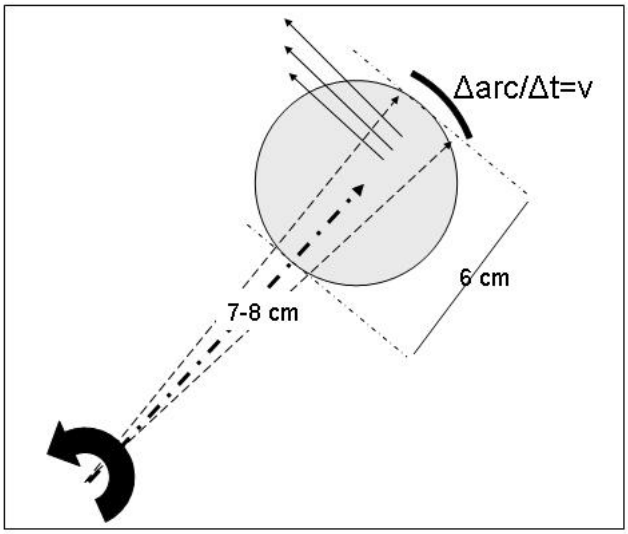

Figure 1. Endothelial cells were culture in Petri dishes of 6 cm in diameter, located in a rotating platform of 24 cm diameter; within the platform, local values of the tangential velocity augments as radial distance increases.

The mesh was generated using the acuMeshSim program, which also facilitated the generation of very refined boundary layer elements from the dish surface. The resultant model consisted of over 755,000 tetrahedra elements, and 137,000 nodes. The CFD solver used in this work was ACUSOLVE CFD from ACUSIM Sofware Inc., Mountview Ca., code based on a special stabilized Galerkin/Least Squares (GLS) finite element technology formulation of the full transient 3D non-linear Navier-Stokes equations of fluid dynamics. The fluid was modeled primarily as a newtonian fluid with the nominal properties of water.

Protein secretion assays. Released vWF antigen was measured in samples taken from the culture supernatant of the Petri dishes using a sandwich immunoenzymatic assay detailed by Morga-Ramírez (2007).

Glycosylation assays. In order to investigate the effect of low Re rotational agitation on the glycosylation patterns of vWF, we measured the total concentration of trimannose and terminal sialic acid resides on vWF released by confluent cultures of endothelial cells agitated at different rotational speeds. Mannose and sialic acid content was calculated by an enzyme lynked lectin assay (ELLA) slightly modified from that reported by Collados *et al.* (1999).

RESULTS AND DISCUSION

Analysis of local velocities. Although geometrically simple, and laminar in nature, the flow system analyzed here is highly complex. Interestingly, a wide range of local velocities can be imposed to the cultured cells by varying the rotational speed of the platform or the radial distance at which the center of the Petri dish is positioned. Cells located in the vicinity of the platform perimeter experience local speeds as high as 5 m/s (8.38 cm/s) when rotated at 8

RPM. Lower local velocity values are observable as we approach the center of the rotating platform or as the rotational velocity decreases. This range of local velocities translates into a range of Reynolds number values (Re) from 0 to 100, still well into the laminar regime. Also, Γ_{xy}, the maximum normal shear stress exerted by the inertial momentum of the culture media over the layer of cells growing at the bottom surface of the Petri dishes, can be estimated by the equation: Γ_{xy} (dynas/cm^2)= -μ dv_x/dy. In this equation, v_x is the velocity component tangential to the cell layer and y is the distance in the direction normal to the flow. The value (dv_x/dy) is the strain rate due to the velocity component in the x direction (tangential to the bottom of the Petri dish). Consequently, the shear stress felt by the monolayer of endothelial cells growing at the bottom of the Petri dish (consider that the cell monolayer is 10 microns height), ranges from 60 to 0 dynes/cm^2. A more comprehensive and realistic characterization of the 3D velocity field, the flow patterns, and the shear stresses induced by the platform rotation inside the Petri dishes is feasible using CFD (Computational Fluid Dynamics) analysis.

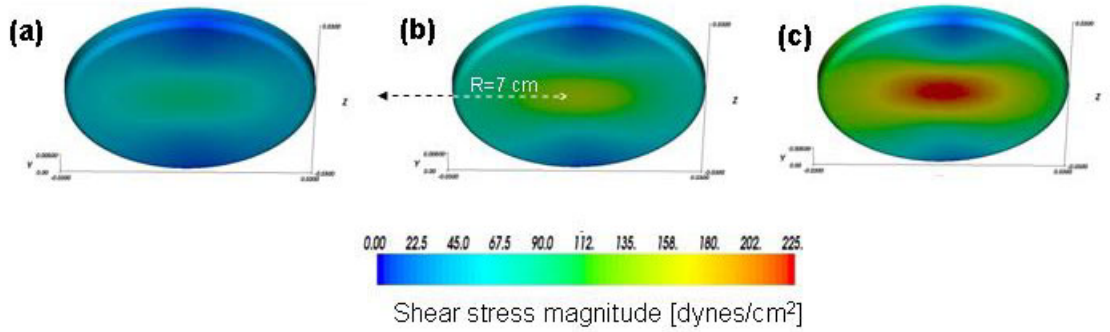

Figure 2. Shear stress values calculated by CFD at 0.05 cm from the bottom surface of Petri dishes rotating at (a) 3 RPM, (b) 5 RPM, and (c) 8 RPM.

Figure 2 presents CFD calculations of the strain rate fields prevalent just above the layer of anchored cells. For a Newtonian system, the shear stress is proportional to the shear rate, being the corresponding proportionality constant the fluid viscosity. If we consider that $v_y \bullet 0$ (equivalent to state that the vertical velocity component is very small compared to the radial and tangential velocity component) then (Γ_{xy}) at each point of the cell monolayer can be approximated by the product of the viscosity times the strain rate calculated by CFD. Moreover, since the typical range of viscosities of mammalian cell culture media approximately averages 1 cp, shear stress values are approximately equivalent to the strain rate values. The highest shear zone is again located at the central areas of each Petri dish. However, CFD analysis reveals very interesting, heterogeneous and non expected shear stress spatial distributions. Globally, maximum shear rates above 50 dynes/cm^2 occur at the central area of dishes rotated at 8 RPM. In the culture systems rotating at 3 and 5 RPM, maximum shear rates do not exceed 40 dynes/cm^2. Subsequently, we will review the effects of these complex flow and shear stress fields in the level of secretion and glycosylation extent of HUVEC cells under culture.

Effect of agitation on von Willebrand secretion levels. Galbucera *et al.* (1997) grew human endothelial cells in a cone and plate system, a well studied flow device, under laminar flow conditions. In these experiments, the cell monolayer was exposed to shear stress values also from 0-12 dynes/cm^2. They found that the secretion of the von Willebrand factor was modulated by the shear stress. As shear stress augments, the von Willebrand concentrations in the extra-cellular environment increased proportionally. In our experiments, we explored a wider range of shear stress values (0-60 dynes/cm^2). In average, rotational speeds as low as 3 RPM (Re•30; Γ_{xy}•30.0 dynes/cm^2) stimulate cells to increase the release of vWF concentration 2.44 fold with respect to non agitated conditions. At 5 RRM (Re•50; Γ_{xy}•37 dynes/cm^2) a statistically similar secretion value was observed with respect to 3 RPM experiments. At 8 RPM (Re•80.0; Γ_{xy}•55.0 dynes/cm^2), the excreted vWF concentration was, in average, 3.21 times that observed in static experiments. Our results are consistent with those reported by Galbucera *et al.*, (1997) for the low Re regime (Re•30; Γ_{xy}•30.0 dynes/cm^2). However, for the range of Re>50 (ranged not explored before) we found progressively lower vWF secretion levels, as the rotational speed increases.

Effect of agitation on Von Willebrand glycosylation. Glycosylation is a highly complex cellular process, dependant on the 3-dimensional structure of the protein, the enzyme repertoire of the host cell, the transit time in the Golgi and the availability of intracellular sugar-nucleotide donors (Butler, 2006). A fundamental objective of this communication is to investigate the effect of different rotational protocols on the extent of glycosylation of the vWF secreted by HUEVEC cultures. As elegantly demonstrated by Naismith and Field (1996), Concanavalin A binds trimannosides, specifically the trimannosides core for biantenary N-linked glycans (see figure 3).

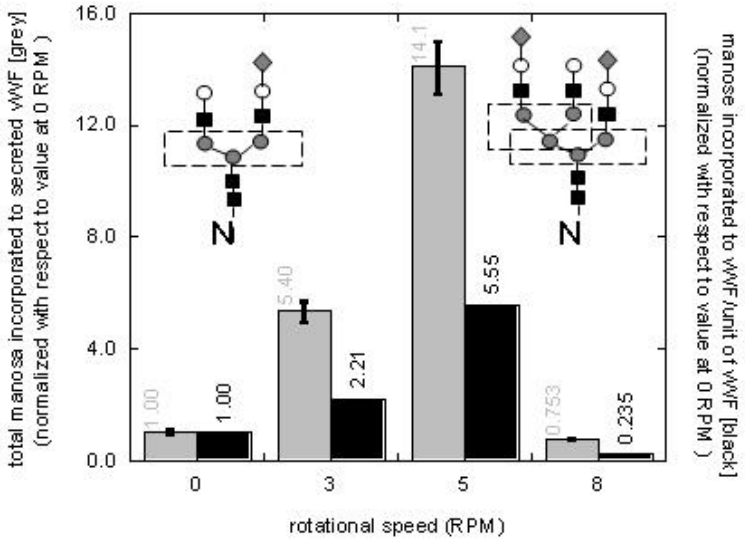

Figure 3. Glycosylation extent at different rotational conditions. Evaluation done by determination of the amount of trimannose core structures using Concanavalin A. Data has been normalized with respect to secretion at static conditions. Each bar presents results from 3 to 4 replicas. Glycans presented in the figure depict location of trimannose cores (within dashed rectangle), common structures to all N-glycan forms.

The fore mentioned authors crystallized the Con A-trimmanoside complex, identifying the Con A binding site and calculating the distances between each one of the three mannoses of the glycan core and the lectin aminoacid residues responsible of the binding specificity. Due to this specificity, Con A binding extent can be an adequate indicator for N-glycosylation extent, since the trimannose core is a common structure among both clases of N-glycans, high-mannose and complex glycans (see for example Manzella *et al.*, 1996).

Figure 3 presents experiments at which Con A is added to confluent HUEVEC cultures to measure glycosylation extend of the secreted vWF. Results have been normalized with respect to the values registered at static conditions (light gray bars). The overall trimannose concentration, as determined by our Con A assay, was dramatically different in Petri dishes rotated at different speeds. Overall trimannose concentration was, in average, 5.40 times higher in dishes rotated at 3 RPM than in static experiments. In 5 RPM experiments, overall trimannose concentration was almost three fold higher than observed at 3 RPM and 14.1 fold that observed in static conditions. Not intuitively, trimannose concentration in Petri dishes rotated at 8 RPM dropped significantly, 25% lower with respect to 0 RPM experiments. To account for different expression levels at each agitation condition, a second normalization, this time with respect to the vWF excreted, is presented (dark gray bars). The extent of glycosylation per molecule also varies significantly between agitated and non-agitated conditions. Remarkably, the ratio of trimannose/vWF molecule secreted in experiments at 3 and 5 RPM is, respectively, 2.21 and 5.55 fold higher than that observed in static cultures. This result made evident that agitation, even at low Re values (•30) can promote dramatic changes in glycosylation activity. Interestingly, in the experiments at 8 RPM, the extent of gliycosylation is significantly lower than that observed in experiments at 5, 3 and 0 RPM (trimannose/vWF= 0.235) making explicit a non-linear and significant dependence of glycosylation extent with respect to agitation.

In addition, we conducted experiments to measure the sialic acid content in vWF as a second indicator of glycosylation quality. Since sialylation is the last intracellular stage of the glycosylation process that takes place in the Golgi, its extent is an indicator of terminal quality of the glycosylation process (see for example Chuan *et al.*, 2006; Shauer 2000, and Gramer *et al.*, 1995). Complete glycosylation of recombinant proteins is usually associated with maximization of galactosylation and sialylation (Butler, 2006). Figure 4 presents sialic acid content of vWF secreted in the supernatant in confluent HUVEC cultures exposed to different rotational velocities, as determined through binding by SNA (a lectin that exhibits high specificity for sialic acid terminal structures). Results are normalized with respect to the values observed for static cultures. Note that the average content of sialic acid determined in experiments at 3 and 5 RPM is, respectively, 6.33 and 7.64 times higher than that observed for static cultures. However, in experiments at 8 RPM, the overall sialic acid content was only 1.40 times that observed in static cultures. If a second normalization is done, now considering that the amount of vWF excreted at different rotational conditions, results from experiments at 3 and 5 RPM are statistically similar (exceeding approximately three fold the values measured for non agitated conditions). Interestingly, in the experiments at 8 RPM, once normalized by the amount of vWF excreted in those experiments, the sialic acid/vWF ratio is lower than that observed in static experiments. At normal conditions, vWF is a highly sialylated glycoprotein. Approximately 56% of their N-linked oligosaccharides are monosialylated and 30% are disialylated (Matsui *et al.*, 1992). Consequently, in average, per glycan molecule linked to vWF,

1.06 sialic acid terminals are present. From an analogous analysis, there are 1.09 trimannose core structures by glycan molecule. Consequently, the expected trimmanose/sialic acid ratio in vWF is 1.03. Our results indicate trimannose/sialic acid ratios of 0.85 and 1.84 for our experiments at 3 and 5 RPM respectively. Therefore, the significant increase in glycosylation extent observed at rotational values of 5 RPM is not accompanied by a proportional sialylation.

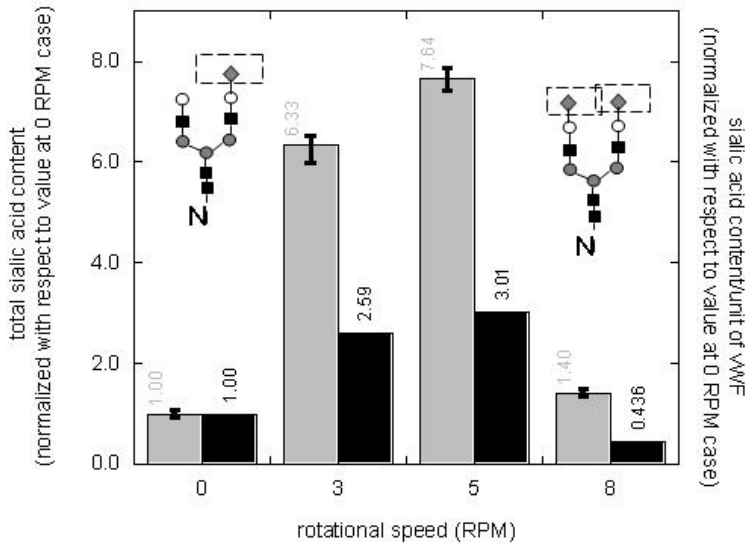

Figure 4. Glycosylation completeness at different rotational conditions, as evaluated by determination of the amount of sialic acid using SNA. Data has been normalized with respect to secretion at static conditions. Each bar presents results from 3 to 5 replicas. Glycans presented in the figure depict location of sialic acid terminal residues (within dashed rectangle). Each glycan might have or not sialic acid residues terminating each antenna.

CONCLUSIONS

Rotational speeds in the low Reynolds regime have significant effect on the levels of secretion and the glycosylation patterns of the von Willebrand factor expressed by HUVEC cultures. This contribution confirms the amplified secretion of vWF observed before in endothelial cell cultures exposed to moderate shear stress levels. Additionally, we document, to our knowledge by the first time, significant changes in glycosylation patterns in the vWF under different shear stress and/or agitation conditions. Our results support the hypothesis that even modest differences in agitation conditions may induce important changes in the secretion and assembly of glycoproteins in mammalian cell cultures.

Our observations might also have relevant implications in the medical context, particularly on the understanding of the complex biochemical cascade related to cardiovascular disorders..

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