Understanding the Geometric Language of Endothelial Cells Under Flow Condition

Comprensión del lenguaje geométrico de las células endoteliales sometidas a flujo

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ABSTRACT

The use of *in vitro* cell culture studies has emerged as an important counterpart to *in vivo* investigations of vascular biology. Cells also depend under geometric and physical laws and tissue engineering has introduced new ways to understand cell behaviour. The study presented here focus on the responses of vascular endothelial cells to fluid frictional stress by using a mathematical and morphological analysis. A flow system was created where we submitted cultures of bovine aortic endothelial cells. We demonstrated that 80% of the endothelial cells under *in vitro* flow conditions align themselves toward the incoming flow. The shape of the cells submitted to the flow is dependant on the flow strength. We showed that the formation of lamellipodia is associated with a 103° angle and we propose that this angle could be the geometrical expression of Rac proteins. We used a new vectorial approach to show cell migration direction. We propose that endothelial cells under flow conditions adopt a shape to optimize their hydrodynamic capacities. With this study, we will advance in our understanding of the cellular geometrical comportment, an important feature of the endothelium artery coverage.

Keywords

Vectorial Approach; Geometric Analysis; Cell Behaviour; Lamellipodia.

RESUMEN

En biología vascular, los estudios con cultivos celulares han emergido como un contrapeso importante para los estudios *in vivo*. La ingeniería tisular ha introducido nuevas vías para entender la conducta celular ya que éstas tiene una dependencia de las leyes de la física y la geometría. El estudio aquí presentado tiene como objetivo evaluar la respuesta de células endoteliales vasculares sometidas a un flujo que produce estrés de fricción, utilizando un análisis matemático y morfológico. Fue creado un sistema de flujo para le evaluación de cultivos celulares de células endoteliales aórticas bovinas. Demostramos que el 80% de las células endoteliales sometidas a flujo se alinean ellas mismas en dirección al mismo. La forma de estas células fue dependiente a la fuerza del flujo. Demostramos que la formación de lamellipodias esta asociados con un ángulo de 103°, y proponemos que este ángulo podría ser la expresión geométrica de las proteínas Rac. Utilizamos un nuevo método que muestra la dirección de la migración celular. Proponemos que las células endoteliales sometidas a flujo adoptan una forma para optimizar sus capacidades hidrodinámicas. Con este estudio, se avanza en el entendimiento del comportamiento geométrico celular, un aspecto importante en el endotelio arterial.

Palabras clave:

Abordaje Vectorial; Análisis Geométrico; Conducta Celular; Lamellipodia.

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Introduction

Recent advances in our understanding of vascular biology have been achieved using both *in vivo* and *in vitro* approaches. A promising new strategy to converge these approaches is the use of tissue engineering to create more physiologic system *in vitro* models. Cell comportment has been extensively studied on a biomolecular basis. However, this description is insufficient to describe the entire cell behaviour that also depends on geometry and physics laws.

Cells form nodes of network through which information circulates. Information can come from close contact via adhesive junction or from far contact via various cell surface receptors [1, 2]. For example, the vascular endothelium, the monolayer of cells lining the inner surfaces of blood vessels is constantly exposed to pressure, stretch, and frictional forces associated with blood flow [3]. The flow plays a central role in vascular pathology, most notably to the endothelium dysfunction associated with early stages of atherosclerosis. The endothelial cells (ECs) monolayer not only provides a selective barrier for macromolecular permeability between the blood and the vessel wall, but also serves a number of important homeostatic functions: vascular remodeling, homeostasis modulation, inflammatory responses and regulates vascular smooth muscle cells contraction [4-6].

Recent development in biochemistry and molecular biology show that cells can interpret, transform, react and transmit incoming signals. We have now reached a point from which we can use this knowledge to communicate simple specific messages to cells. Cell language is at a molecular level but it is modulated by physical conditions. The extracellular domains of integrins bind to specific ligands (extracellular matrix) such as fibronectin, vitronectin and collagen. The cytoplasmic domain interacts with signalling molecules in the focal adhesion sites [7]. The unique structural feature of integrins enable them to mediate both “inside-out” signalling, in which intracellular signals modulate the affinity of integrines for extracellular ligands, and “outside-in” signalling, in which extracelular stimuli induce the intracellular signalling cascade via integrin activation [8]. These high affinity integrins are crucial for cell motility, though dispensable for cell adhesion. On the other hand, the bidimensional geometrical distribution of ligands on the substrate can modulate cell interpretation of the incoming signal as well as three dimensional structure of extracellular matrix, now related to Rac activation [9].

Studies on intracellular signaling events in ECs have shown that shear stress activates multiple signaling molecules, including protein kinase C (PKC), FAK, c-Src, Rho family GTPases, PI3K, and MAPKs [2,4,10,11]. The Rho family of guanine nucleotide (GTP)-binding proteins act as molecular switches, they coordinate the actin cytoskeleton organization and gene transcription [12]. In general, RhoA increases cell contractility, focal adhesions, and actin stress fibers formation; Cdc42 regulates fillipodia formation; Rac regulates membrane ruffling and is well-known for its ability to stimulate actin polymerization at the plasma to form lamellipodia, that are necessary structures for cellular migration [13-21]. It plays a central role in cell migration and spreading through induction of lamellipodia, promoting directed motility of ECs. Specifically stimulates the initial extension therefore prompting (generating) new adhesions [22].

Multicellular organisms are complex networks which use long distance as well as short distance interactions via solubilized factors and via close proteins contacts. Organogenesis depends on determinist parameters such as DNA but also on environmental factors that can interfere with the intercellular communications.

Material and Methods

Flow system

A 75 cm^2 Petry flask (Fisher Scientific, Dreicich, Germany) was pierced on one side with two holes. The middle part of two 5 ml pipettes (Stripettes, Fisher Scientific, Dreicich, Germany) cut in three parts, was introduced in the holes and sealed with silicone. To avoid movement of the pipettes parts, they were attached together on the outside of the flask in a way to maintain a tension between them. Two medical grade rubber tubes (Tube Tygon, R-36-06, K Mac Plastics, Wyoming, MI, USA) were fixed on the pipettes parts and attached together via a bigger tube (Tube Masterflex, 06485-82, Cole-Parmer, Vernon Hills, IL, USA) necessary for the peristaltic pump (Cole-Parmer, 7529-60, Vernon Hills, IL, USA) (Fig. 1), this situation does not create turbulences. The pump was kept outside of the incubator. The rubber tubular hoses were tightly passed through the door side. The inside of the Petry flask was covered with pork gelatine on which the bovine aortic endothelial cells (BAECs) were cultivated until confluence without liquid flux (see section on cell culture for fluid composition). When we reached confluence, a periodic flux was initiated (0.10 l/min) and augmented linearly during three hours (0.15 – 0.20 l/min) to obtain a final flux of 1 l/min. We used three different speeds 2, 6 and 10 m/min. Flux was maintained for 24 h and after that time, cells were fixed and stained for observations.

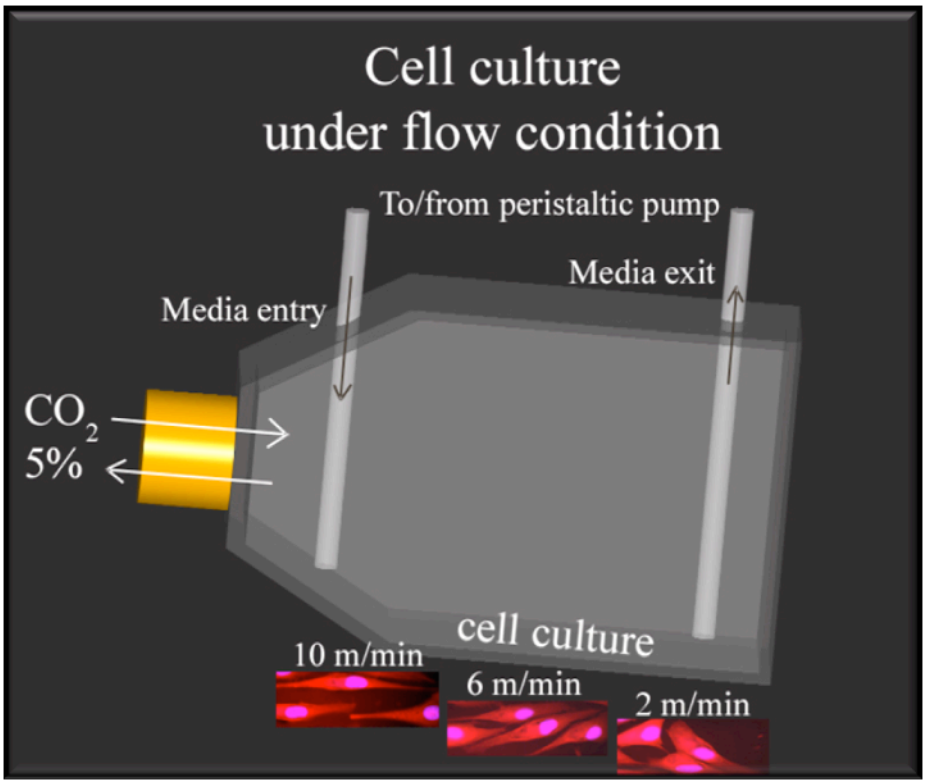

Figure 1. Experimental setup used to grow cells under flowing media.

Cell culture

BAECs (Cambrex BioScience, Walkersville Inc., MD, USA) were cultivated in a 75 cm^2 tissue culture-treated polystyrene flask maintained on the side with two clamps. The cells (n = 500) were kept in a 37 ºC incubator with a humidified atmosphere and 5% CO_2. Culture media was made of Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St Louis, MO,

USA) with 10% veal serum (Hyclone, Logan, UT, USA) added with 100 g/ml of penicillin G, 100 g/ml of streptomycin (Invitrogen Canada Inc., Burlington, ON, Can). The final volume was 50 - 75 ml.

Fixation and staining

DMEM Culture medium (Sigma-Aldrich, St Louis, MO, USA) was suctioned and cells were immersed 30 min at room temperature in 3.7% formaldehyde in PBS 7.4 (Sigma-Aldrich, St Louis, MO, USA). Formaldehyde solution (Sigma-Aldrich, St Louis, MO, USA) was then removed from the wells plate, and cells were washed three times with PBS 7.4. Cell permeability was increased with a solution of PBS pH 7.4 with 3% BSA (Sigma- Aldrich, St Louis, MO, USA) and 0.1% saponin (Sigma-Aldrich, St Louis, MO, USA) for 1 h 30 min at room temperature. This solution was removed and cells were stained using DAPI (6 µg/ml in PBS, Sigma-Aldrich, St Louis, MO, USA) and rhodamine-phalloidin (0.12 µg/ml in PBS 7.4, Sigma-Aldrich, St Louis, MO, USA) and maintained in the 37 ºC incubator for 1 h. Cells were then washed 6 times with PBS and 0.05% tween 20 (Sigma-Aldrich, St Louis, MO, USA) before the surface was fixed between blade and lamella to be observed under the microscope.

Microscopy

Microscopy photographs were taken using a Nikon E800 confocal microscope equipped with a Hamatsu Orca ER digital camera (Nikon Canada, Mississauga ON, Can). Photographs were analyzed using CLEMEX Vision software (CLEMEX Vision, Longueuil, QC, Can) and released Beta 4.0.3 of Scion Image (Copyright 1997-2005, Scion Corporation, Frederick, MD, USA).

Image analysis

DAPI stained nuclei appeared as blue circular shapes. Rhodamine-phalloidin stained actin show as red fibers. Sizes of the surface and photography fields were considered as being constants. Aligned cells were counted on nine photographs with three different conditions corresponding to normal and extreme limits of *in vivo* blood speed (2, 6 and 10 l/min). The number of flow aligned cells was compared to the number of unaligned cells. Length and width of cells were measured using the longest axis in both longitudinal and transversal directions. Lengths were measured in pixels and then transformed in µm with the photograph at scale. Angles and lengths were measured with the Beta 4.0.3 of Scion Image software (Scion Corporation, Frederick, MD, USA). The number of trials to ensure reproducibility of experiments was two (n = 2). Experiments were in duplicate and were repeated twice.

A new vectorial approach to show cell behaviours

We associated a vector to observable fillipodia. The length and the direction of the vectors were associated to the length and direction of the fillipodia. A displacement must result of a reaction-counter reaction between two masses. So, assuming that the cytoskeleton is attached to focal contacts, it is possible to determine the direction of migration of the cells on the surface by adding each vector following the classical vector summation:

$$\text{Equation. 1:} \quad V = \sum_i u_i + v_i$$

where u_i and v_i are the two dimensional components of each vector i.

Mathematical regression from bidimensional images for characterisation of cells shapes

To characterise the cell shape we traced the external layer of the cells on a Cartesian plot with 30 to 40 dots on each side. We then fit the curve with a fourth or fifth order regression depending of the regression coefficient (R^2) obtained. We assumed that a R^2 between 0,85 and 1,00 was close enough to be a good representation of the cell shape. We used the equations for further computational analysis with Maple 7 (Maplesoft, Waterloo, ON, Can) such has to find the second order differential to determine the position of the inflexion point and the first order differential to quantify the gradient of divergence between the two curves (Fig. 2).

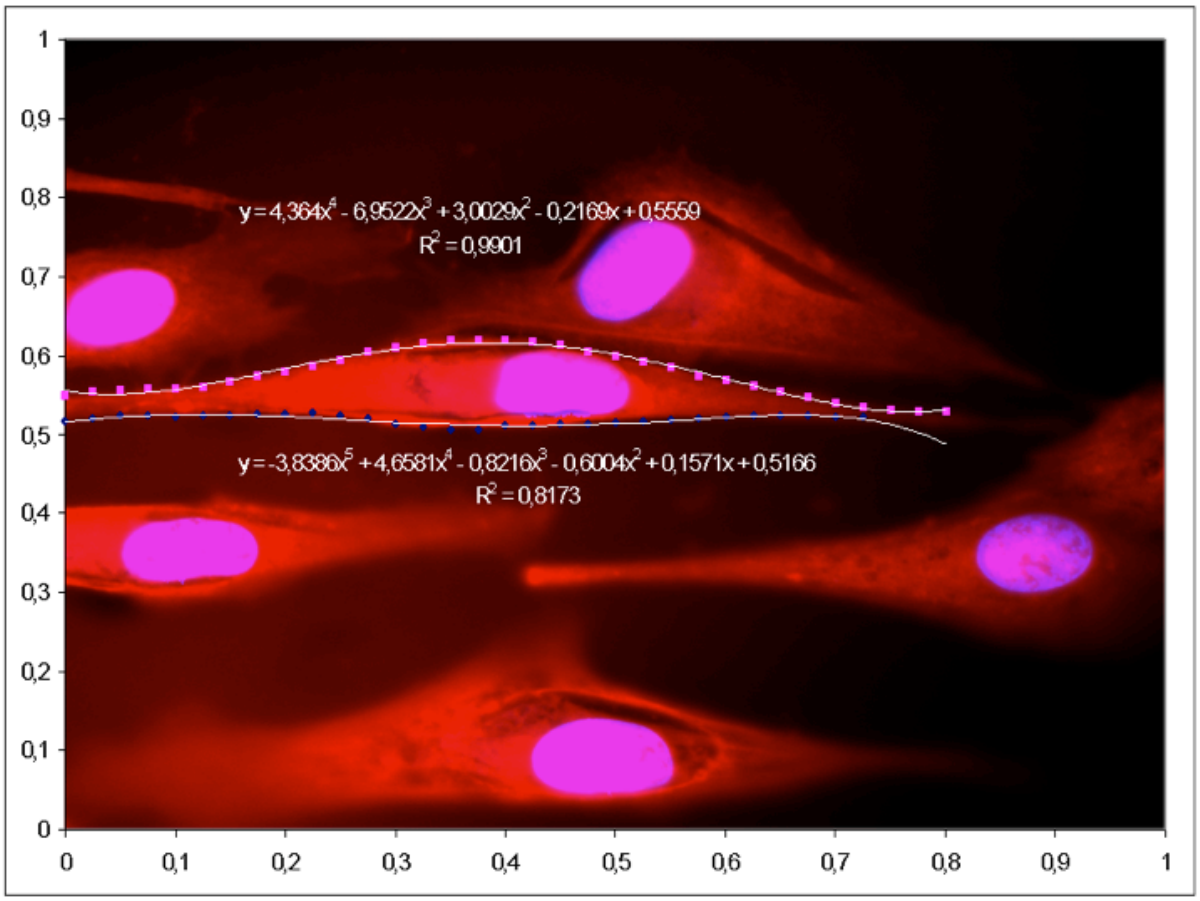

Figure 2. Fourth and fifth order regression to model external cell shape.
An equation is associated to the cell surface for further mathematic analysis.

Results

Three different speed flows gave three different cell shapes (Table 1). These three flows represent three different conditions: hypotension (2 m/min), normal tension (6 l/min) and hypertension (10 l/min). Cells under a flow of 10 ± 1 l/min were the longest with an average length of 115 ± 2 µm (S = 10.7). However the lengths of cells under a flow of 6 and 2 l/min did not change and were both of 73 ± 2 µm (S = 8.5). Width of cells varied from 14±2 µm for a speed flow of 2 ± 1 l/min (S = 3.7), then reduce to 8 ± 2 µm for a speed flow of 6 ± 1 l/min

(S = 2.4) that unexpectedly increased to 10 ± 2 µm with an increase of speed flow to 10 ± 1 l/min (S = 3.1) (Fig. 3).

The number of aligned cell is also dependent on the flow velocity. We counted 85 ± 3 % (S = 9.2), 71 ± 3% (S = 8.4) and 57 ± 3% (S = 7.5) of aligned cells under speed flows respectively of 10 ± 1 l/min, 6 ± 1 l/min and 2 ± 1 l/min. However, cells in low speed media show a bigger amount of diploid cells associated to proliferation or dividing cells: respectively 2.66%, 2.11% and 1.49% for 2, 6 and 10 l/min.

Flow (±1 m/min)	*Average cell length (±2 µm)*	*Average cell width (±2 µm)*	*Ratio length/width (±2 µm)*	*Ratio of aligned cells (±3 %)*
10	**115**	**10**	**12**	**85**
6	**73**	**8**	**9**	**71**
2	**73**	**14**	**5**	**57**

Table 1. Cells proportions in relation with medium flow.

Morphologic analysis of cells permitted to point out certain characteristics of cell behavior and cell geometry. Figure 4 shows four different cellular behaviors observable with rhodamin-phalloidin markers. First, in red, are underlined some small fillipodia associated to lamellipode zones with a 130° angle. We observed the same angle on nine different photographs. Most of them are facing upstream. The formation of these lamellipodia is liable of cell alignment toward liquid flow. A second cell activity is marked with a yellow pentagon and shows a zone of big intracellular activity. This particular sector contains bright points of fluorescent rhodamin-phalloidin marker suggesting a high-density zone of actin activation. This rebuilding of the cytoskeleton is necessary to permit the cell to turn around and face the flow stream. Aligned cells leave a space between them. This room has almost a perfect shape for entering another cell in between, even when no cell is present (white dashed line). This observation means that the mosaic structure of formed *in-vivo* endothelium is an intrinsic consequence of the blood flow. This shape is not induced by cell-cell contacts but depend on the blood displacement.

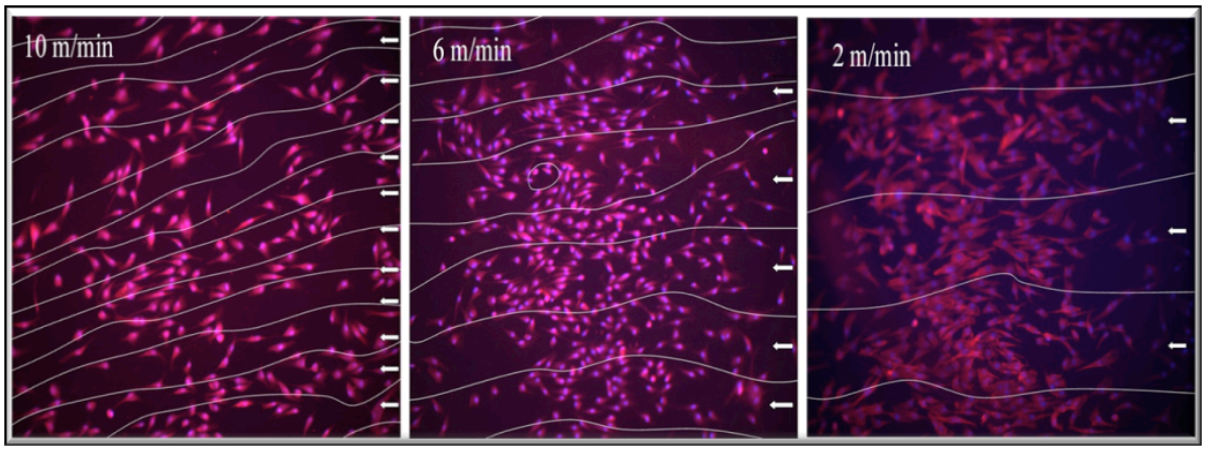

Figure 3. Cell cultures under 10, 6 and 2 m/min flux under 10 x magnifications.

An interesting feature of observed fillipodia is the convergence of their prolongation to a particular zone. We propose that the position of emerging fillipodia might be controlled by the position of these converging zones. The quasi absence of emerging fillipodia on the other side of the cell permits to affirm that the emergence of the fillipodia is related to the direction of the stream. The stream exerts strength on the cell membrane that is transmitted to the surface via focal contact.

Finally Figure 4 shows that the end of the cell is formed with a large stress fiber. This structure is very resistant and permits to other cell to get a strong pull upstream.

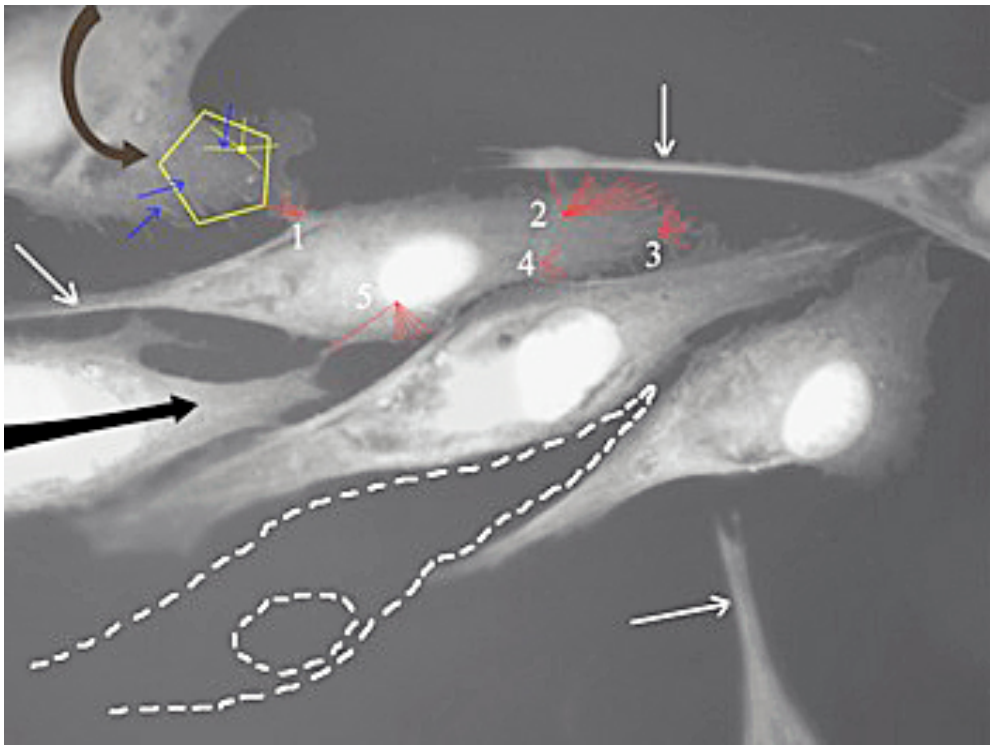

Figure 4. Cell morphology related to flow and cytoskeleton proteins. 1,2,3,4 and 5 are actin fibers converging points (red) limited in a 103° angle. Yellow

pentagon illustrates a zone of intense remodeling of cell cytosqueleton for the cell to turn around and get aligned to the flow. Blue arrows point toward actin stress fibers. Black arrows illustrate global cell movement. White arrows show big long stress fibers related to cell's tail. Dashed line illustrates a space into which another cell could get park. White lines represent the schematic image of an endothelial cell.

Discussion

The major finding of this study is the geometrical analysis of endothelial cells under different flow conditions. This study was repeated twice and validate. The cells under higher flow velocity were significantly elongated and aligned with the flow direction and the lengths of these cells had a significant increment (42 ± 2 mm) in relation with the other cells. In contrast, the width of these cells was bigger than the cells under flow velocity of 6 l/min. We presume that the length of the fiber is related to its function. Longer fibers are degrading however shorter are polymerizing. In this point of view it is possible to associate vectors to cytosqueleton fibers. Each vector (linear fiber) corresponds to a force exerted on organelles of the cell (nucleus, membrane, etc). Displacement of the cell is thus related to vector exerting force on particular zones of the cell membrane called focal contacts. The analysis of cell geometry permitted to point out that under flow conditions, cells exert force against the current stream.

Migration of endothelial cells is critical for the formation of new blood vessels during angiogenesis. Different studies have also identified a role of Rac in producing and increasing in ECs stress fibers and focal adhesions and this production of these cystoskeletal features through Rac proteins increase motility [23]. In this research we observe in the lamellipodia territory the formation of 103° angles, it is the first time that this angle is demonstrated and we are sure that it could be the geometric representation of Rac because this protein is involved in the lamellipodia formation. This angle was the result of the fork of different prolongations of the lamellipodia area. The formation of these lamellipodia is liable of cell alignment toward liquid flow. We consider that this discovery is very important since the cell is geometry, for what we should begin to know the mathematical language used by the cells. Actually, we know that the actin activation express a 70° angle. For what we are convinced that most of the proteins have a geometric expression that we should know.

In conclusion, with a new vectorial approach, we demonstrated that liquid flow orient cells and induce different cell geometry. That flow rate influences cell proliferation, as reflected in the range of cell division. However, variations in speed flow have not a linear effect on cell geometry. We established the basics of a new computational method for cell characterisation which permits to associate lamellipodia with a 103° angle. We propose that this angle could be related to the expression of Rac proteins. We are convinced that if we have enough geometric information, we will begin the process of understanding the mathematical language used by the ECs, with the purpose of detecting in a simple image if the cell is in a good state or is suffering. This is important for arterial prostheses. We think that the mathematical language of the cell will be used in a not very distant future as a diagnostic and prediction index.

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Glossary

R^2: when the regression line is linear (y = ax + b) the regression coefficient is the constant (a) that represents the rate of change of one variable (y) as a function of changes in the other (x); it is the slope of the regression line.

S: summation operator

Integrins: are large modular cell-surface receptors that regulate almost every aspect of cellular function through bidirectional signals transmitted across the lipid bilayer.

Shear Stress: a parallel frictional biomechanical drag force that is determined by blood flow, vessel geometry and fluid viscosity that is computationally estimated using fluid dynamics models and is expressed in units of dynes/cm^2.