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Characterization of hemodynamic *ex vivo* model to study endothelial activation by TNF-α in prefunded human veins

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Abstract

Inflammation is recognized as part of the etiology of numerous diseases. The interaction among cells of the immunological system with local cells and molecules, such as cytokines and chemokines, allows cellular activation and response amplification. The importance of several physicochemical factors like frictional force, vascular flow, shear stress, and pressure is now recognized because they are known to modulate genetic expression and endothelial activation; however, there are very few studies that recreate such cellular microenvironments. Hence, it is of paramount importance to develop new models that will mimic physiological conditions. Our aim was to improve a human vein ex vivo model that would allow endothelial activation in flow conditions, to study the molecular components during adhesion, taking into consideration physicochemical parameters such as flow and shear stress. Endothelial umbilical human vein was used and activated with TNF-α in order to determine U937 monocytic cells adhesion, as well as cytokines secretion and ICAM-1 expression. This model will allow leukocyte adhesion studies, using different inflammatory stimulus, along with the signaling pathways involved in several pathologies. (Gac Med Mex. 2015;151:192-201)

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Introduction

Inflammatory processes are currently recognized as part of the etiology of a large variety of chronic degenerative diseases, including diabetes and cancer. This type of processes involve not only tissue-resident leukocytes and monocytes, but also other types of cells such as fibroblasts, epithelial cells, muscle cells and endothelial cells and, therefore, understanding the interaction of immune system cells with its microenvironment has become necessary to understand the inflammatory

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process at the cellular and molecular level[1,2]. In this context, leukocyte adhesion to the vascular endothelium is essentiol to the migration of different cell-types to the site of injury or infection. Currently, different molecules involved in this procedure have been already identified, such as growth factors, chemokines and cytokines, with the vascular endothelial growth factor (VEGF), interleukin 8 (IL-8), MIP-1α, TNF-α, interleukin 1β (IL-1β) and interferon γ (INF-γ) standing out, as well as adhesion molecules such as selectins, integrins, immunoglobulins, cadherins, etc.[3-5]. However, it is important mentioning that very few of the current studies on leukocyte adhesion and communication with endothelium consider certain important factors such as vascular flow, which plays an essential role in this type of phenomena.

Vascular flow, frictional forces and pressure are important in endothelial processes such as vasodilation, passage of nutrients and coagulation, even in the formation of nanoparticles and in inflammatory focuses in some diseases, such as atherosclerosis and lupus[6,7]. In the past few years, these physical factors have been shown to be able to modulate endothelial gene expression. Frictional forces have been shown to be able to alter the expression of genes such as *p53,* which entails the arrest of cell-cycle in neutrophils[8], or proteins such as Hur, involved in the regulation of molecules such as TLR4 and ICAM-1[9]. Thus, changes in flow velocity can favor or affect leukocyte adhesion by changing endothelial morphology and activation[10,11].

Acute or chronic inflammation has traditionally been studied in *in vitro* models by employing peripheral blood cell lines or leukocytes, or *in vivo,* using ummunosuppressed or transgenic rodents[12,13], but most of these models fail to consider the above mentioned vascular flow and frictional force parameters. Some studies with monolayer-grown human cells subjected to flow conditions have found expression of molecules other than those from the endothelium inside the vein[14]. Furthermore, murine models have demonstrated that not all endothelial cells have the same capacity to bind leukocytes, even when they express adhesion molecules, since they have been shown to be able to express different molecules, depending on the involved cells[15]. This is true with ICAM-1, supported by the receptor for advanced glycosylation end-products (RAGE), which maintains neutrophil adhesion *in vivo*[16-18].

In view of all this, there is an emerging need for the design of new models that allow for the molecular components that regulate adhesion under flow conditions inside human veins to be studied. The purpose of this work was to design an *ex vivo* human vein model that would allow for the human endothelium to be activated under flow conditions in small veins, in order to enable the study of molecular components of adhesion, taking into account flow and frictional physicochemical parametrs.

For this, the human umbilical vein endothelium, which was activated with TNF-α, was used as a model, in order to be able to determine circulating U937 cells adhesion and some of the molecular components associated with this event.

Material and methods

Acquisition of cells and tissues

Umbilical veins were obtained from uncomplicated eutocic deliveries or c-sections at the Hospital of Obstetrics and Gynecology "Dr. Luis Castelazo Ayala" from the Mexican Institute of Social Security (IMSS – *Instituto Mexicano del Seguro Social)*, in Mexico City, following a clinical protocol previously approved by the Ethics Committee of the hospital. Once collected, the veins were divided into 5-cm sections, in order to apply different treatments with the same length. The veins were placed in containers with PBS supplemented with antibiotic-antimycotic (1.5%). The time from the collection of the specimen to the start of the experiment never exceeded 3 h.

The U937 promonocytic leukemia cell-line was obtained from ATCC (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (6408-02) (Cole -Parmer Chicago, Il, USA) supplemented with 10% of fetal bovine serum (GIBCO-BRL, Rockville, MD, USA), 1% of non-essential amino acids, penicillin 100 U/ml and streptomycin 100 µg/ml (GIBCO-BRL, Rockville, MD, USA); supplemented media are referred here as complete media. The medium was substituted every 2 or 3 days. The cells were cultured at 37 °C in a 5% CO_2 athmosphere.

Perfusion system

The perfusion system was designed using double-bottom glass containers (Imparlab, Mexico City, Mexico), to allow for water to flow (water bath, Poly-Science, Illinois, USA). Perfusion tubes were of 3 mm in diameter for the RPMI-1640 perfusion medium and were coupled to the glass reservoirs. Flow pressure and velocity conditions were controlled using a peristaltic pump (Masterflex 7015, Cole-Parmer Chicago, IL, USA).

Determination of frictional and flow forces

To determine the frictional forces and type of flow (laminar or turbulent) within the vein during the adhesion assays, the viscosity (μ) and density (ρ) values of the completed media in the absence and presence of cells had to be established. For the assays, 30 ml of supplemented RPMI-1640, with or without U937 cells (3 x 10^6 cells/ml) were employed. Viscosity of the RPMI-1640 complete medium was determined using a PSV002AB rheometer (Brockfield Engineering laboratories, Inc., USA) and the dependence of viscosity as a function of temperture was analyzed. Angular velocity was 100 g from 10 aliquots taken during 2 min, 20 s each one from 8 to 30 °C. Data were captured using the Rheovision 32 v.2.0 program and were analyzed with Origen v.7.0. Mean density of the RPMI-1640 medium was determined by weighing 1 ml of medium at 25 °C and it was 1,007 g/ml with fetal bovine serum; these values were used to obtain the Reynolds number ($RN = \rho Dv/\mu$)[19]. The obtained value was $RN \leq 2{,}000$, indicating that flow velocity within the vein behaves as a laminar flow. The used flow was 900 ± 100 μl/min, which is the physiological velocity in 1 mm-diameter human venules. Frictional force was calculated using the equation for Newtonian flows: $\tau = 6\mu Q/h2w = (6\mu/h2w)\ Q$, where t is the frictional force or shear stress (dynes/cm^2); μ, the viscosity coefficient of the fluid, h, the height of the vein; w, the width of the vein, and Q, flow velocity[20]; and these parameters were fixed at $\tau = 1.8 \pm 0.2$ and $\tau = 2.2 \pm 0.2$ dynes/cm^2 with and without circulating U937 cells, respectively.

Histochemistry and immunofluorescence assays

To observe the anatomy of the human vein perfused from 0 to 6 h in hemodynamic conditions, perfusioned veins were sectioned and fixed in isopentane (sigma Aldrich, USA), to then make 7-μ sections with a cryostat (CM-18950, Leica Systems, USA), which were later frozen at -20 °C. Stainings were performed with hematoxylin/eosin (HE), and the samples were observed using a light microscope Axioscop40 (Carl Zeiss, Jena, Germany). Simultaneously, stainings for immunofluorescence were performed with specific antibodies with affinity to the components of the vascular structure, such as Von Willebrand Factor or β-sarcoglycans; this was accomplished with anti-mouse and rabbit primary antibodies (Santa Cruz, CA, USA) diluted 1:100 and then coupled with Alexa Fluor 647 secondary antibodies (Molecular Probes, Oregon, USA). The samples were observed using a LSM-510 confocal microscope (Carl Zeiss, Jena, Germany).

Lactate dehydrogenase assay (LDH)

Membrane damage and induced cell death during the assays were determined by the amount of LDH released in the perfusion medium after 1-h U937 cells recirculation in the human vein, either pre-activated for 2 h with TNF-α at a 5 mg/ml concentration or without stimulus. As the control, the obtained value was compared with that of released LDH in the static culture medium (without recirculation) of human umbilical vein endothelial cells (HUVEC) under the same conditions. In both cases, the samples were centrifuged for 5 min at 100 x g and frozen at –70 °C until their determination. LDH activity was quantified using the methodology described by Barry et al. (1991), by using NADH (0.24 mM) and pyruvate (9.76 mM) as substrates, and absorbance at 340 nm was quantified every 15 s during 2 min in a DU 640 spectrometer (Beckman, USA). Additionally, the viability of recirculating and static U937 cells after perfusing them for 2 h was determined using the trypan blue assay. A 1 ml aliquot was taken every 5 min in order to count live cells. The cells were incubated with a tryptan blue solution at 0.4% (Life tech, USA) for 7 min and then washed 3 times with PBS for 1 min. Live and dead cells were counted in a Neubauer chamber and the result was reported in percentages with regard to the 100% of U937 live cells at the initial time.

Measurement of nitric oxide (NO) production

To assess the endothelial response to physiological stimuli in our model, the concentration of NO was measured in the perfusion media and the supernatant of the cultured cells. NO was determined using a colorimetric assay based on the Griess reaction. The nitrite/nitrate ratio in the perfusion medium was determined and quantified as a surrogate measure of the produced NO in response to TNF-α activation. The equation of the curve was obtained by least square regression and was used to calculate the concentration values in the samples. A standard curve ranging from 1 to 5 nM of KNO_3 was constructed, and a linear regression of R2 = 0.997 was obtained.

Determination of cytokines with ELISA testing

TNF-α-stimulated and non-stimulated veins, perfused for 2 h, were used; once perfusion was concluded, the vein was washed for 10 min and placed in RPMI medium, again with and without recirculating U937 cells (1×10^6 cells/ml) for 1 h; after this time was elapsed, the media were collected and frozen at –70 °C until their utilization. The human cytokine ELISA Ultrasensitive 10 panel plex kit (Invitrogen, USA) was used. For the cytokines concentration determination, a standard curve was created to interpolate the results of the samples, as recommended by the kit. The concentration was reported in pg/ml.

Adhesion assays

Endothelial activation was determined after TNF-α-activation in flow conditions by measuring U937 monocytic cells adhesion. The TNF-α concentration selected to be used in the *ex vivo* assays was obtained from previous experiments where a TNF-a concentration curve was created ranging from 0.5 to 10 ng/ml, in a *in vitro* adhesion assay of U937 cells to HUVECs. The concentration with the highest adhesion was 5 ng/ml. At the start of the experiments, the system was washed with 2% of Extran MA biologic detergent for 10 min and, finally, the system was washed again with sterile PBS for 10 min. At the beginning of the experiment, two 5-cm segments from a single umbilical cord were used; both segments were placed in the lower reservoirs. One vein segment was activated with 5 ng/ml of TNF-α in RPMI medium, while the other was perfused with medium without TNF-α. Flow velocity was established at 1 ml/ml at 37 °C for 2 h. When this time was elapsed, the veins were washed with PBS for 10 min. The U937 cells were labeled with carboxyfluorescein succinimidyl ester (CFSE). Approximately 1×10^7 U937 cells were incubated with 5 nM of CFSE in PBS supplemented with 0.5% of bovine serum albumin at 37 °C for 15 min. Then, the cells were washed twice with RPMI medium and were centrifuged for 5 min at 700 x g. The cells were resuspended in complete medium and placed in the container for the perfusion of the human veins for 1 h. After perfusion, the veins were washed for 10 min with PBS and were frosen in cold isopentane to be stored at –70 °C until cryostat slicing (CM-18950 Leica Systems, USA) into 7-µ thick cross-sectional sections. Cell adhesion was assessed by counting the number of cells on the cell surface in images of the vessel's lumen reported in mm^2 in 25 separate camps. Observation was made in a LSM-510 epifluorescence microscope (Carl Zeiss, Jena, Germany.

Data analysis

Data are presented as the means ± SD of five independent experiments carried out in triplicate. One-way ANOVA tests, followed by the Tukey-Kramer analysis of variance, were used to compare the samples. A p-value lower than 0.05 was considered to be statistically significant.

Results

Assembly of the perfusion system and assessment of umbilical vein integrity under flow conditions

The design of our system is based on hepatic perfusion models, but it was adapted to the size of human umbilical cord veins and to the employed medium volume. The diagram on figure 1 A indicates the minimal components it comprises and the flow sense of a peristaltic pump. Figure 1 B shows the perfusion system of the human vein, which comprises two reservoirs that allow for independent perfusion of two umbilical cord separate segments. A flow controller allows for the passage of medium coming from the upper reservoirs to be controlled. Lower reservoirs maintain the cord at 37 °C and a sterile venoclysis system serves for the circulation of the medium.

Identification of the perfused vein structure for this system was assessed using HE staining. Figure 1 C shows HE representative images where the three consecutive layers that comprise a vein are observed: tunica intima (TI), tunica media (TM) and tunica adventitia (TA). Using the immunofluorescense technique, the Von Willebrand factor, which is endothelial-cell specific, was labelled (Fig. 1 D); the Hoechst-labelled nuclei are observed in blue, whereas the presence of smooth muscle cells of the vein was visualized with red-stained β sarcoglycans labelling. These results showed that this model allows for vascular architecture to be preserved after perfusion, which is very important for subsequent adhesion assays.

In addition to vein architecture, cell damage by effect of flow in perfused veins, whether TNF-α-perfused or not, was assessed. The results presented in table 1 demonstrate that only 15% of LDH is released by perfused veins when not activated. This value was

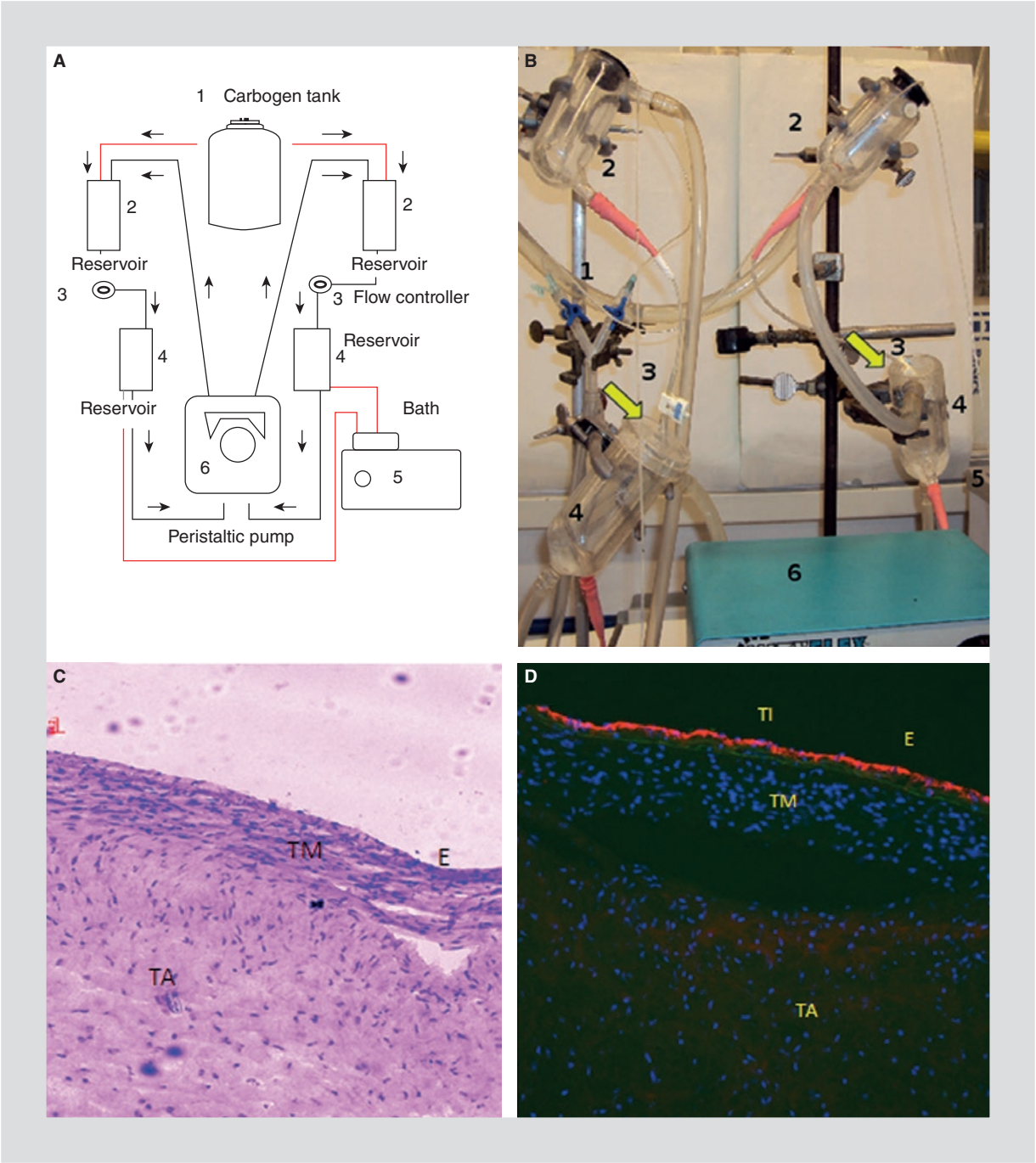

Figure 1. *System for human umbilical vein perfusion.* **A:** *diagram of the work system; arrows indicate the flow sense.* **B:** *glass reservoirs for the medium and the vein; arrows indicate the place where the human vein is placed.* **C:** *veins were stained with HE, as described in the "Material and methods" section, to label the components of the perfused veins.* **D:** *the immunofluorescense technique was used to label the Von Willebrand factor, which is endothelial cell-specific. The nuclei were labeled with Hoechst and the smooth muscle cells of the vein were visualized with β-sarcoglycans labeling.* **E:** *endothelium.*

similar to that obtained for HUVEC cultures without perfusion and higher than those obtained for TNF-α-activated veins and non-perfused, but activated cultures. This shows that perfusion does not affect vascular endothelium membrane's integrity, whereas activated endothelium better resists cell-damage. In the experiments where U937 cells were recirculated to perfused veins or non-activated HUVEC cultures (third and fourth rows), the LDH level in the medium increased to 38.2 and 11.4%, respectively. The results for activated

Table 1. Cell integrity determination by LDH release in perfused human veins in comparison with cell cultures without perfusion. Percentage of released LDH with regard to 100% of each cell-type lisated with Triton X-100 is indicated. Standard deviation is shown for each result

Cell type	LDH released without TNF-α stimulation (%)	LDH released in TNF-α-stimulated cells (%)
HUVEC	15.4 ± 0.1	3.3 ± 0.1*
Human umbilical vein	14.7 ± 6.1	5.87 ± 0.9*
HUVEC + U937	11.4 ± 3.3	11.2 ± 2.1
Human umbilical vein + U937	38.2 ± 2.4	38.1 ± 2.2†

*Significant data with regard to control with no TNF-α.
†Significant differences with regard to non-perfused U937 cells (ANOVA $p < 0.05$).

veins and HUVECs were very similar, and differences in cell death were only found with U937 cells present, but not by endothelial activation. The increase in the release of LDH in media where U937 cells were recirculated can be interpreted as damage of these cells by perfusion, and not by endothelial damage. Therefore, and in order to decrease cell damage and to verify the U937 cells viability by effect of circulation, the number of times that cells passed through the peristaltic pump was reduced from 4 to 2. Additionally, the volume of perfusion media was increased from 9 to 12 ml, in order to avoid changes in the volume of flow passing through the vein each complete cycle. These changes reduced integrity and death of the U937 cells, thus maintaining cell viability above 90% (Fig. 2) after 2.5 h. These conditions were used for the adhesion assays.

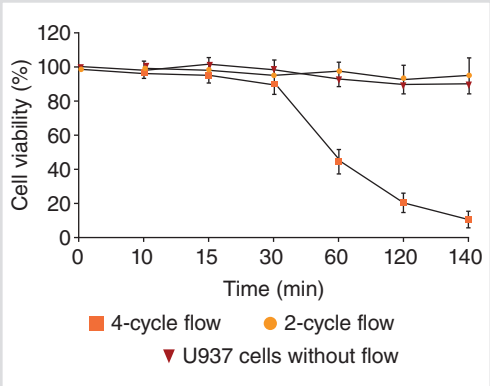

Figure 2. *Viability of perfused U937 cells. Either U937 cells were recirculated by the system 2 and 4 times at 1 ml/min for 140 min, or the reservoirs were kept without perfusion. Subsequently, aliquots were taken to determine viability by staining with tryptan blue. Bars show standard deviations for each samnple (ANOVA $p < 0.05$).*

Measurement of endothelial response under flow conditions

Indirect determination of NO production

To verify if vascular endothelium was capable to respond to inflammatory stimuli, the production of NO and pro-inflammatiry cytokines was quantified. The standard curve for nitrite/nitrate quantification showed a linear range of 5 to 50 μM of KNO_3, with a linear regression coefficient $r^2 = 0.997$. Nitrite/nitrate concentration baseline index in the medium of the perfused vein was 23.24 mm ± 6.7. The perfusion medium of veins treated with 5 ng/ml of TNF-α showed a significant increase of 50% ($p < 0.05$), since it was 39.27 ± 4.1. In addition, the production of inflammatory cytokines under flow conditions by human veins with and without TNF-α and in the presence or absence of U937 cells, during 2-h activation and 1-h U937 cells circulation, was quantified. The ELISA cytokine analysis in the perfusion medium of activated veins showed a significant pg/ml increase in the production of higher levels of TNF-α, as well as IL-4, GM-CSF, IL-6 and IL-8, with regard to non-stimulated veins under flow conditions (Fig. 3). The media of TNF-α-stimulated veins without circulating cells released IL-8 and TNF-α after 2 h of vein activation and 1 additional h and without cells in comparison with non-stimulated veins.

Cell adhesion assay

Adhesion was quantified by assessing U937 cells on the endothelial surface at the vessel lumen of TNF-α-

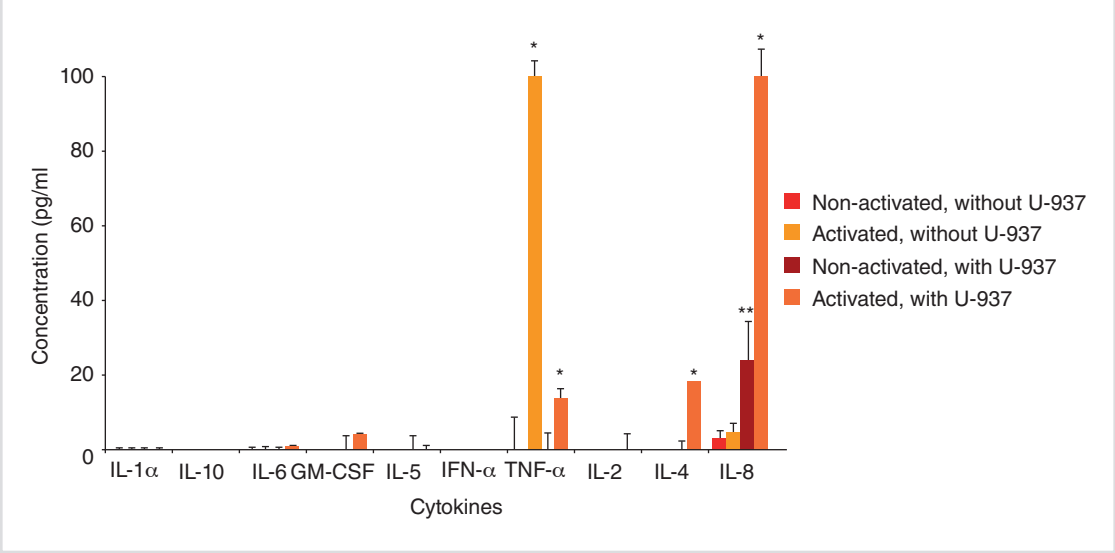

Figure 3. *Multiplex-assay for cytokines produced by activated veins. Indicated are veins treated by TNF perfusion for 2 h and veins with subsequent U937 cells circulation for 1 h more. After each treatment, veins were washed with PBS for 10 min. The asterisk (*) shows significance with regard to the presence of that cytokine in non-stimulated veins and without circulating cells. Double asterisks (**) indicate significance for the non-activated veins with U937 cells with regard to non-activated veins and without U937 cells ($p < 0.05$).*

treated and non-treated veins (Fig. 4). The results showed a significant 7.1-fold increase in the number of U937 cells adhered in perfused veins activated with TNF-α (175.71 ± 12.3 cells/mm^2) in comparison with non-activated veins (15 ± 6.9 cells/mm^2). Subsequently, veins were also activated for 0.5 h and 1 h, and U937 cells were quantified after 1 h of perfusion, without showing significant changes in adhesion. TNF-α-activated and non-activated veins for 0.5, 1, 2 and 2.5 h were fixed and stained by immunofuorescence to assess the ICAM-1 expression. The results are presented in figure 5. Changes are visible in the presence of ICAM-1 after 2 h

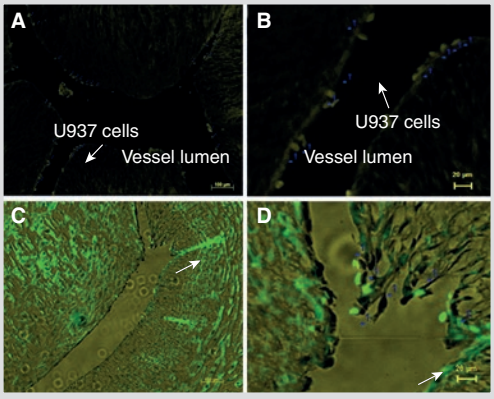

Figure 4. *Adhesion assay and U937 cell quantification on the vascular endothelium.* **A** *and* **C***: vessel lumen in a human vein section without stimulation with TNF-α.* **B** *and* **D:** *enlargement of the above images to evidence the U937 cells on the endothelial surface. Arrows point at U937 cells.*

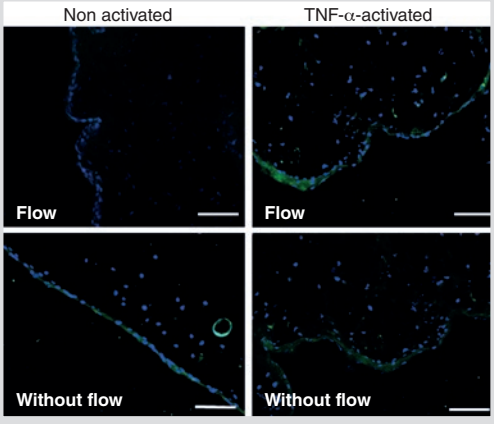

Figure 5. *ICAM-1 expression in TNF-α-activated veins in the presence or absence of flow. The figure shows cross-sectionally sectioned veins, which were labeled with anti-ICAM-1 and anti-mouse Alexa-488. The bar indicates the distance (400 μm).*

in the activated endothelium in comparison with perfused veins without the cytokine and with regard to veins treated for shorter periods (data not shown).

Discussion

In this work, a new *ex vivo* perfusion system that simulates laminar flow conditions and the presence of vascular flow, equivalent to that found in human umbilical cord venules, was developed. For this, our first objective was the preservation of tissue viability, as well as those of vascular structure and system architecture. Our results show that umbilical vein viability percentage remains within ranges reported for other perfusion models in saphenous veins and aorta[21,22], which have reported that cell viability is preserved from 1 h to 5 days, with endohelial functionality being maintained. For the purposes of this work, tissue viability and integrity was required to be maintained for a lapse of time no longer than 5 h, but the model allows for time to be increased for other type of assays. To that end, reservoirs should simply have to be hermetically sealed, thus avoiding possible contamination of the medium passing through the vein.

The use of the umbilical vein method allows for human tissues to be analyzed but, since thes tissues are alive, the conditions are closer to the biological context and provide with a model for the conduction of experimental research on human diseases, in particular for acute diseases, in time intervals ranging from minutes to hours or days. Conversely, murine models for studies in chronic inflammation are used in studies with ranges as long as months, but have the disadvantage of using genetically or physiologically modified animals (immunosuppressed, immunodeficient or transgenic) and fail to reproduce cellular and molecular mechanisms that occur in human vessels. The umbilical vein maintains a differenciated morphology, such as that of small veins or the aorta in adults and, from the functional point of view, it responds to the same factors than veins and arteries, such as biomechanical or inflammatory stimuli[23,24]. This means that they also express surface molecules that are characteristic from the endothelium and respond to mechanical stimuli such as pressure, type of flow and frictional forces.

As previously mentioned, hemodynamic forces are essential for leukocyte adhesion; hence, pressure and frictional forces have recently been paid attention to, since both can modulate different aspects of endothelial cells physiology and morphology[25,26]. These forces, along with flow velocity and type of medium, can also regulate the expression of different genes related to coagulation, vasodilation and inflammation, thus locally or systemically affecting the endothelial phenotype. In general, it is uncommon to find studies in static models using this type of parameters during assessment of endothelial activation in the inflammatory process, such as cultures of endothelial cells obtained from umbilical cord or HUVEC[29-29], and only in the past few years they have started to be considered for the study of endothelial activation and leukocyte adhesion[30,31].

The model here presented responds to stimuli that affect vasodilation and vasoconstriction, such as activation of the inducible nitric oxide synthase (iNOS) enzyme, which is activated by TNF-α during the inflammatory process. Similarly, the activated endothelium responds via the signalling pathway of the transcription factor NFkB to induce the production of cytokines and chemokines like TNF-α itself or INF-γ, IL-1β, IL-8 and MIP-1α, among others, through the activation of TNF-α, as demonstrated in the perfused veins of our model.

U937 monocytic cells are known to adhere to the endothelium only after previous activation and, therefore, the type of stimulus will depend on microenvironmental factors (the vein) facilitating their adhesion. Having a model where hemodynamical conditions can be fixed to maintain the flow enables to analyze whether it favors or restricts endothelial activation, not only by the chemical or cellular stimulation it generates, but also by the presence of rheological stress on the employed circulating cells.

Our *ex vivo* model achieved to significantly increase the adhesive capacity of the endothelium to bind U937 monocytic cells at a 1 ml/min flow after activation with TNF-α, which strongly suggests that the NFkB-controlled classical inflammation pathway, which has already been demonstrated *in vitro,* becomes activated. However, it is possible for other pathways to participate, as flow can induce endothelial changes that might activate alternative pathways, such as the AP-1 or STAT-3 pathways, since we observed less adhesion than with assays in static monolayers, but a higher number of monocytic cells adhered with each other and to endothelial cells. The fact of having found less U937 cell adhesion to the endothelium, unlike assays in monolayers, may be due to the delay in contact between different adherent cells, or else to the delay in the binding of the recombinant TNF-α to its surface receptor, due to the presence of vascular flow; however, the fact of having found an increase in

the IL-8 cytokine in the presence of U937 cells, suggests an activation of these cells to favor adhesion, with STAT-3 being one of the pathways that might become activated[6,32,33].

In addition to all that has been mentioned until this moment, another known important factor for vein-circulating monocytes, macrophages, lymphocytes or polymorphonuclear cells adhesion during inflammation is the proximity to the activated endothelium in the site of damage or disease and their physical interaction with adhesion molecules. Initially, a loose adhesion to the endothelium takes place thanks to molecules such as selectins, but as time passes, the expression of molecules of the immunoglobulin family, such as VCAM-1, PECAM-1 and ICAM-1, allows for more firm adhesion that favors leukocyte extravasation. However, the effect of vascular flow on the inflammatory microenvironment of human veins remains unknown, since it is not clear whether the expression and adhesion of these proteins require less or more involved molecules, or if these events occur by activation of the same signalling pathways reported in different *in vitro* or murine models. This implies that the timing of leukocyte adhesion to the endothelial surface start and termination do not have to be the same as those in monolayer flow models, which fail to reproduce vessels endothelial morphology of vessels[34,35].

In this work, the adhesion molecule ICAM-1 was labeled in samples of vessels activated with adhered cells and a greater presence of ICAM-1 was observed in the activated endothelium; however, this molecule was not always associated with zones with adhered cells. ICAM-1 expression in different zones of the endothelium where no adhered U937 cells are observed may be an indicator that, in the presence of vascular flow, endothelial activation can occur either in cells that can come to be in contact with the monocytic cells, or in those that respond to changes in the type of flow (from vascular to turbulent), as has been reported in murine models[36-38]. Determining the change in ICAM-1 expression during U937 monocytes adhesion in a time curve (0.5, 1, 2, 3, 6 h), as well as the expression of other molecules such as selectin E and VCAM-1 would be very interesting in order to know how do these molecules respond to flow changes during loose and/or firm adhesion in the endothelium *ex vivo.*

Finally, it is relevant mentioning that we have managed to establish a human umbilical vein *ex vivo* system that preserves endothelial integrity and viability and that might be used to analyze other cell-adhesion molecules associated with endothelial activation of other molecules, such as platelets, to assess endothelial adhesiveness changes under hemodynamical conditions.

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