Video Article

Isolation of Human Umbilical Vein Endothelial Cells and Their Use in the Study of Neutrophil Transmigration Under Flow Conditions

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Abstract

Neutrophils are the most abundant type of white blood cell. They form an essential part of the innate immune system[1]. During acute inflammation, neutrophils are the first inflammatory cells to migrate to the site of injury. Recruitment of neutrophils to an injury site is a stepwise process that includes first, dilation of blood vessels to increase blood flow; second, microvascular structural changes and escape of plasma proteins from the bloodstream; third, rolling, adhesion and transmigration of the neutrophil across the endothelium; and fourth accumulation of neutrophils at the site of injury[2,3]. A wide array of *in vivo* and *in vitro* methods has evolved to enable the study of these processes[4]. This method focuses on neutrophil transmigration across human endothelial cells.

One popular method for examining the molecular processes involved in neutrophil transmigration utilizes human neutrophils interacting with primary human umbilical vein endothelial cells (HUVEC)[5]. Neutrophil isolation has been described visually elsewhere[6]; thus this article will show the method for isolation of HUVEC. Once isolated and grown to confluence, endothelial cells are activated resulting in the upregulation of adhesion and activation molecules. For example, activation of endothelial cells with cytokines like TNF-α results in increased E-selectin and IL-8 expression[7]. E-selectin mediates capture and rolling of neutrophils and IL-8 mediates activation and firm adhesion of neutrophils. After adhesion neutrophils transmigrate. Transmigration can occur paracellularly (through endothelial cell junctions) or transcellularly (through the endothelial cell itself). In most cases, these interactions occur under flow conditions found in the vasculature[7,8].

The parallel plate flow chamber is a widely used system that mimics the hydrodynamic shear stresses found *in vivo* and enables the study of neutrophil recruitment under flow condition *in vitro*[9,10]. Several companies produce parallel plate flow chambers and each have advantages and disadvantages. If fluorescent imaging is needed, glass or an optically similar polymer needs to be used. Endothelial cells do not grow well on glass.

Here we present an easy and rapid method for phase-contrast, DIC and fluorescent imaging of neutrophil transmigration using a low volume ibidi channel slide made of a polymer that supports the rapid adhesion and growth of human endothelial cells and has optical qualities that are comparable to glass. In this method, endothelial cells were grown and stimulated in an ibidi µslide. Neutrophils were introduced under flow conditions and transmigration was assessed. Fluorescent imaging of the junctions enabled real-time determination of the extent of paracellular versus transcellular transmigration.

Video Link

The video component of this article can be found at https://www.jove.com/video/4032/

Protocol

1. Isolation and Maintenance of Human Vascular Endothelial Cells

1. Level 2 biosafety procedures must be used when working with human blood and tissue. Using scissors cut the cord from the placenta and then closely examine the cord for blood clots and damage caused by clamping the cord during delivery. Cut out and discard these portions of the cord.
2. Prepare fresh collagenase solution at 1 mg/ml by pouring 50 ml of warm cord buffer into a tube that contains 50 mg collagenase.
3. Change into sterile gloves and open an autoclaved instrument tray containing sterilized blunt cannulas, cord clamps, scissors, three-way stop-cocks and gauze in a laminar flow hood.
4. Identify the two arteries and the single vein present in the cord. The arteries are smaller and tend to be tightly constricted, whereas the vein is large and easy to cannulate. To flush the blood from the vein, gently insert a cannula with a two-way stopcock attached to it into one end of the vein and place a clamp around cannula to hold it firmly in position. Perfuse cord buffer through the vein. Repeat until flow through is clear and then clamp the end of the cord.

5. Perfuse collagenase into the vein, close the stopcock, place the cord into a beaker containing warm cord buffer and incubate for 10 minutes. After 10 minutes, remove the cord and gently massage the cord to loosen endothelial cells from the lumen of vein. Drain the solution into the tube containing endothelial cell media (ECM). Flush with cord buffer twice to remove remaining cells. Drain the solution into the same tube.
6. Centrifuge the cells at 350 x g for 10 minutes to pellet the endothelial cells.
7. Remove the supernatant and add 10 ml of ECM to the pellet. Gently resuspend the cells in ECM, then transfer the cells to a T75 flask pre-coated with 0.2% gelatin. Pre-coating with gelatin has been done for at least 1 hour at 37 °C. Grow the cells at 37 °C and 5% CO_2.
8. The next day, gently shake the flask to dislodge any red blood cells then remove the supernatant and wash with warm Hank's balanced salt solution (HBSS). Remove HBSS and feed the cells with 10 ml of warm ECM. Examine the cells under the microscope to ensure that the red blood cells were removed. Evaluate the degree of confluence and morphology of the endothelial cells. Endothelial cells at this stage should be elongated with no sign of vacuoles.
9. Continue to change the media every three days until cells reach confluency, then split cells using a 0.08% solution of trypsin containing 1mM EDTA. Plate endothelial cells in desired dishes pre-coated with 0.2% gelatin. Cells should reach confluence in 3-6 days.
10. When using ibidi chambers, pre-coat each chamber with 0.2% gelatin. Remove excess gelatin and fibronectin and then add 1.5 X 10^6/mL of endothelial cells suspended in ECM into the chamber. Change the media every other day until confluent. Cells should be confluent in 2-3 days depending on the cord.

2. Preparation of Neutrophils

1. Neutrophils were isolated from the peripheral blood of healthy human volunteers using density centrifugation. A detailed protocol with a visual demonstration for neutrophil isolation can be found in the earlier issue of this journal [6]. Once isolated, resuspend neutrophils at a concentration of 1x10^6/mL in HBSS containing 0.5% human albumin.

3. Setting up the Ibidi Chamber

1. Grow endothelial cells in a gelatin-coated ibidi chamber. Examine them every day using phase contrast microscopy until they become tightly confluent.
2. Stimulate the endothelial cells to increase the expression of adhesion and activation molecules. For the purposes of this protocol, endothelial cells were stimulated with 10 ng/mL of recombinant TNF-α for four hours at 37 °C and 5% CO_2 prior to setting up the ibidi chamber for imaging under flow conditions.
3. Prepare the microscope, and syringe pump as visually described by Wiese and colleagues [11] using an empty ibidi chamber.
4. Key differences between this protocol and the Wiese procedure include pre-filling the tubing to prevent exposure of the endothelial cells to air, maintaining the buffer at 37 °C using a water bath, using PharMed tubing to keep the buffer from losing temperature and using different objectives on the microscope depending on whether imaging with phase contrast or fluorescence.
5. Once the microscope is set up and the tubing is filled with HBSS, close all the stopcocks and remove the empty ibidi chamber.
6. Connect the ibidi chamber containing TNF-α stimulated endothelial cells to the tubing.

4. Neutrophil Recruitment and Transmigration

1. Visualize the endothelial cell monolayer in the microscope using a 10x phase contrast objective.
2. Set the syringe pump to withdraw and begin the flow of HBSS at the desired shear stress (typically between 0.5 to 2 dyn/cm^2).
3. On the inlet side, switch from HBSS to isolated neutrophils (1x10^6/mL) by turning the three-way stop-cock.
4. Begin recording using a CCD camera connected either to a DVD recorder or connected directly to the computer. Start the timer when neutrophils enter the chamber. Perfuse neutrophils for four minutes. A total of 3 mL of the neutrophil suspension is sufficient for a single experiment.
5. After four minutes, switch back to HBSS to prevent the attachment of new neutrophils. If data for total interactions, rolling and firm adhesion are desired, image 6 random fields for 10 seconds each using a 10x phase contrast objective between minutes 4 and 5.
6. Switch to a 40x objective and record a single field of view between 5 and 10 minutes. At 10 minutes, collect between 5 and 10 random fields of view.
7. Stop the flow and move to next chamber.

5. Fluorescent Imaging Under Flow Conditions Using an Ibidi Chamber

1. Label either endothelial cells or neutrophils with a fluorescent probe of interest. For example, an anti-VE-cadherin antibody conjugated to Alexa-[547] can be used to visualize endothelial cell junctions.
2. Assemble ibidi chamber on a microscope with differential interference contrast (DIC) and fluorescent capabilities. We use a FluoView 1000 confocal (Olympus) with an environmental chamber. Focus using an objective designed for DIC and fluorescent work.
3. Acquire simultaneous DIC and fluorescent images using software supplied by vendor while following the time course described in section 4.

6. Analysis of Neutrophil Recruitment

1. Analysis of rolling and interacting cells is described by Wiese *et al*[11].
2. To measure the percentage of neutrophils rolling on the endothelial monolayer count the numbers of neutrophils that have moved more than one cell diameter in a period of 5 seconds. Divide this number by the total numbers of leukocytes in the field to determine the percentage of rolling cells. By extension, the remaining cells are considered firmly adherent.
3. Measure the rolling velocity of neutrophil by calculating the distance the neutrophil travelled in a particular time period and then dividing it by that time in seconds.

4. Transmigration is determined by counting the number of neutrophils that have changed shape and migrated beneath the monolayer[10]. These neutrophils are identified by the fact that they change from being phase bright when on top of the monolayer to being phase dark when underneath the monolayer[10]. The number of cells transmigrated can be expressed as a percentage of the total cells in the field of view or as a raw number of transmigrated cells per unit area. In this model system, typically 50% of neutrophils transmigrate after 10 minutes.

7. Representative Results

Unstimulated endothelial cells do not support neutrophil recruitment. In contrast, stimulating endothelial cells with TNF-α results in neutrophil rolling, firm adhesion and transmigration. An example of these data is shown in **Figures 1 and 2**. **Figure 1A** shows neutrophils interacting with TNF-α-stimulated endothelial cells. These interactions can be quantified, revealing the total number of neutrophils interacting with the endothelial cells as well as the number of neutrophils rolling, firmly adherent or transmigrated (**Figure 2**). Neutrophil transmigration can occur at cell junctions or through the endothelial cells themselves. To differentiate between these two conditions, endothelial cells are labeled with an anti-VE-cadherin antibody and transmigration is scored as occurring paracellularly or transcellularly (**Figure 1B and C**). In this model, virtually all transmigration is paracellular[7].

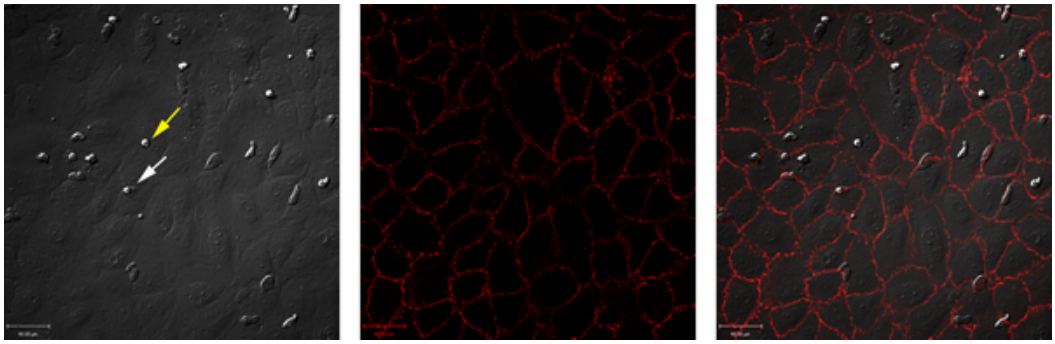

Figure 1. Simultaneous DIC and fluorescence imaging under flow conditions using an ibidi chamber. (A) DIC image freshly isolated human neutrophils migrating across human umbilical vein endothelial cells. The yellow arrowhead shows an adherent neutrophil; the white arrowhead shows a transmigrating neutrophil. (B) Endothelial cell junctions were stained with an anti-VE-cadherin antibody conjugated to Alexa-[547] and imaged. (C) Shows overlay of channel A and B revealing that virtually all transmigrating in this model is paracellular. Click here to view larger figure.

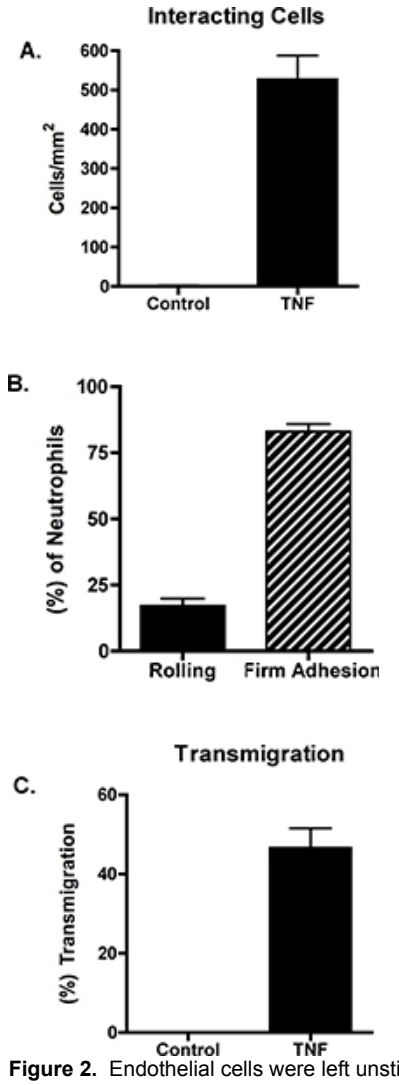

Figure 2. Endothelial cells were left unstimulated or were stimulated with 10 ng/mL of TNF-α. After 4 hours, a parallel plate flow chamber was assembled and neutrophils were perfused across at 1 dyn/cm^2. (A) Neutrophil interactions were examined and measured using ImageJ software from NIH. The total interacting cells were characterized as (B) rolling, firmly adherent or (C) transmigrating. Data represent the mean + SEM of between 3 and 5 experiments. The difference between control and TNF in panels A and C was significant ($p<0.001$).

Discussion

Researchers have routinely used endothelial cells from different vascular beds to study neutrophil recruitment and transmigration. Examples include, but are not limited to, dermal microvascular endothelial cells[12], liver sinusoidal endothelial cells[13] and endothelial cells from human umbilical vein[10]. Among them HUVEC gained wide popularity because of their relative ease of isolation and availability. HUVEC are a powerful *in vitro* model system that mimics many endothelial processes that occur *in vivo*. This model system has aided in the identification of adhesion molecules and chemokines critical in the recruitment of many leukocyte subclasses and enabled detailed analysis of how these processes are regulated. *In vitro* work with HUVEC has formed the foundation for *in vivo* studies that ultimately have provided a better understanding and treatment for human disease[14].

In this paper we present a rapid and simple method to study neutrophil recruitment *in vitro* using an ibidi flow chamber. This kind of chamber has several advantages over other parallel plate chambers described earlier in this journal[11]. Ibidi chambers are made up of a polymer with optical characteristics similar to glass that enables the user to take fluorescent and DIC images in addition to phase-contrast images. There are chambers that use glass coverslips to enable fluorescent imaging, but growing endothelial cells on glass is a challenge as the adhesion property of these cells change significantly on glass surfaces[15]. Endothelial cells adhere well to plastic surface but fluorescence imaging on plastic surface is not achievable. Using the ibidi μ slides not only eliminates these problems but also they require much less sample volume than the other methods. These slides also enable simultaneous parallel experiments with only slight modification.

Disclosures

No conflicts of interest declared.

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