Video Article

Assessment of the Anticoagulant and Anti-inflammatory Properties of Endothelial Cells Using 3D Cell Culture and Non-anticoagulated Whole Blood

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Abstract

In vivo, endothelial cells are crucial for the natural anticoagulation of circulating blood. Consequently, endothelial cell activation leads to blood coagulation. This phenomenon is observed in many clinical situations, like organ transplantation in the presence of pre-formed anti-donor antibodies, including xenotransplantation, as well as in ischemia/reperfusion injury. In order to reduce animal experimentation according to the 3R standards (reduction, replacement and refinement), *in vitro* models to study the effect of endothelial cell activation on blood coagulation would be highly desirable. However, common flatbed systems of endothelial cell culture provide a surface-to-volume ratio of 1 - 5 cm^2 of endothelium per mL of blood, which is not sufficient for natural, endothelial-mediated anticoagulation. Culturing endothelial cells on microcarrier beads may increase the surface-to-volume ratio to 40 - 160 cm^2/mL. This increased ratio is sufficient to ensure the "natural" anticoagulation of whole blood, so that the use of anticoagulants can be avoided. Here an *in vitro* microcarrier-based system is described to study the effects of genetic modification of porcine endothelial cells on coagulation of whole, non-anticoagulated human blood. In the described assay, primary porcine aortic endothelial cells, either wild type (WT) or transgenic for human CD46 and thrombomodulin, were grown on microcarrier beads and then exposed to freshly drawn non-anticoagulated human blood. This model allows for the measurement and quantification of cytokine release as well as activation markers of complement and coagulation in the blood plasma. In addition, imaging of activated endothelial cell and deposition of immunoglobulins, complement- and coagulation proteins on the endothelialized beads were performed by confocal microscopy. This assay can also be used to test drugs which are supposed to prevent endothelial cell activation and, thus, coagulation. On top of its potential to reduce the number of animals used for such investigations, the described assay is easy to perform and consistently reproducible.

Video Link

The video component of this article can be found at https://www.jove.com/video/56227/

Introduction

The vascular endothelium consists of a monolayer of endothelial cells (EC) which line the lumen of blood vessels. In a physiological state, quiescent EC are responsible for the maintenance of an anticoagulant and anti-inflammatory environment.[1] This is mediated by the expression of anticoagulant and anti-inflammatory proteins on the EC surface. For example, EC activation caused by ischemia/reperfusion injury or vascular rejection of (xeno-)transplanted organs results in a change of the endothelial surface from an anticoagulant and anti-inflammatory state to a pro-coagulant and pro-inflammatory state.[1]

To study the fascinating and complex interaction between the endothelium and coagulation factors, *in vitro* models which mimic as closely as possible the *in vivo* situation are highly desirable. A common limitation which characterizes conventional *in vitro* coagulation assays is the use of anticoagulated blood which makes the analysis of coagulation-mediated effects arduous and even recalcification of citrated whole blood cannot reproduce results obtainable with fresh non-anticoagulated blood.[2] Besides, in traditional flat-bed cell culture systems it is impossible to exploit the anticoagulant properties of the endothelium as a sufficient endothelial cell surface per blood volume cannot be reached. The model presented here overcomes these limitations by culturing EC on the surface of spherical microcarrier beads, so that an EC surface-to-blood ratio of >16 cm^2/mL can be reached, which is similar to the situation in small arterioles or veins, and which was described to be sufficient to allow "natural" anticoagulation of the blood by the EC surface.[3,4] Whole blood can be used without added anticoagulants in this setting. Blood samples can be collected during the experiment and cytokines, coagulation factors and soluble complement activation markers can be detected and quantified. Furthermore, EC-coated microcarrier beads may be analyzed for complement and immunoglobulin deposition as well as the expression of EC activation markers by confocal microscopy. Another interesting application includes the testing of drugs which are supposed to prevent endothelial cell activation and, thus, coagulation.[5] Although this model cannot completely replace animal experimentation, it offers a

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method to test specific functional hypotheses *ex vivo* using cells and thus reduce the number of animals used in basic research on ischemia/reperfusion injury or (xeno)transplantation.

The described model was used to mimic a xenotransplantation setting in which porcine aortic endothelial cells (PAEC) are grown on the microcarrier beads and incubated with whole, non-anticoagulated human blood. Different transgenic PAEC, carrying several human genes such as CD46 for the regulation of the complement system and/or thrombomodulin (hTBM) for the regulation of the coagulation system, were analyzed for their anticoagulant properties. Endothelial cell activation, complement, and coagulation systems are tightly controlled and interconnected.[6] It is therefore important to understand how the different transgenic cells behave after exposure to human blood with regard to adhesion molecule expression and cytokine release, shedding of the glycocalyx and loss of anticoagulant proteins.[7]

Protocol

German Landrace pigs (wild type bred in a local farmhouse and transgenic bred at the Institute of Molecular Animal Breeding and Biotechnology, Ludwig-Maximilian University, Munich, Germany), weighing between 30 kg to 40 kg, were used in this study. All animals were housed under standard conditions with water and food *ad lib*. All animal experiments were performed in accordance with the U.K. Animals Act (scientific procedures) and the NIH Guide for the Care and Use of Laboratory Animals, as well as the Swiss animal protection law. All animal studies complied with the ARRIVE guidelines. The animal experimentation committee of the cantonal veterinary service (Canton of Bern, Switzerland) approved all animal procedures, permission no. BE70/14. Experimental protocols were refined according to the 3R principles and state-of-the-art anesthesia and pain management were used to minimize the number of animals and reduce the exposure of the animals to stress and pain during the experiments.

Blood was drawn from healthy individuals by closed system venipuncture in accordance with Swiss jurisdiction and ethics guidelines of the Bern University Hospital. The phrase "non-anticoagulated blood" means that the blood has not been treated with any anticoagulant.

The following steps are performed under sterile conditions. Familiarity with basic cell culture sterile technique is required.

1. Isolation of PAEC

NOTE: Thoracic aorta segments of 6 to 10 cm were obtained from euthanized German Landrace pigs of 3 to 6 months of age (used for other *in vivo* experiments) and immediately transferred into a 500-mL glass bottle containing transport medium (DMEM + 1% penicillin/streptomycin).

1. Pre-coat a 6-well plate with fibronectin 12.5 µg/mL in PBS 1x and place it in an incubator at 37 °C for 1 h.
2. Pre-warm sterile PBS 1x and cell culture medium (DMEM).
3. Take out the porcine aorta from transport medium.
4. Place the aorta on a polystyrene plate.
5. Flush with warm PBS gently beforehand.
6. Cut the aorta longitudinally and fix it with needles.
7. Add warm cell culture medium on the inner vessel surface.
8. Aspirate fibronectin-cell culture medium and add fresh cell culture medium (DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 0.4% of Endothelial Cell Growth Medium Supplement Mix).
9. Soak one cotton swab in the cell culture medium. Swab the cotton wool bud on the very top of the inner vessel surface gently and slowly in the same direction.
10. Rub the cells in one well of 6-well plate round by round.
11. Do the same for the rest of the wells.
12. Check cells under the microscope and place the 6-well plate in incubator at 37 °C, 5% CO_2.
13. Change the medium on the second day and change it again every 2 - 3 days.
14. When cells are going to be confluent, trypsinize cells and seed them into a T75 flask (PAEC P1).

2. PAEC Characterization

1. Pre-coat an 8-well chamberslide with fibronectin 12.5 µg/mL in PBS 1x and place it in an incubator at 37 °C for 1 h.
2. Seed 5 x 10^4 cells/well and incubate overnight in the incubator at 37 °C.
3. Wash the cells twice with PBS^{++}(PBS supplemented with $CaCl_2$ and $MgCl_2$), 300 µL/well.
4. Fix cells with 3.7% paraformaldehyde for 10 min at room temperature, 200 µL/well.
5. Wash cells 3 times with PBS^{++}, 300 µL/well.
6. Add 300 µL of PBS 1x-3% BSA (blocking buffer) and leave 30 min at room temperature.
7. Apply primary antibodies (anti-VE-cadherin, anti-CD31, anti-vWF) diluted in PBS 1x-1%BSA-0.05% detergent, 160 µL/well and incubate for 1 h at room temperature.
8. Wash 3 times with PBS^{++} (200 µL/well).
9. Apply secondary antibodies and DAPI diluted in PBS 1x-1%BSA-0.05% detergent, 160 µL/well, and incubate for 1 h at room temperature.
10. Wash 3 times with PBS^{++} (200 µL/well).
11. Mount slides with glycerol based mounting medium and verify endothelial cell markers expression under a fluorescence microscope.
    NOTE: Culture porcine aortic endothelial cells in a T175 flask (DMEM low glucose medium + 10% FBS, 1% penicillin/streptomycin and 0.4% of Endothelial Cell Growth Medium Supplement Mix) until 90% confluence is reached. (seeding density 1 x 10^6 cells, 90% confluence correspond roughly to 5 x 10^6 cells).

3. Coating of Microcarrier Beads

1. Mix 7 mL of microcarrier beads with 42 mL of collagen solution in a 50-mL tube (100 µg/mL, diluted in a 0.2% acetic acid solution) and incubate for 1 h at room temperature.
2. Wash beads two times with 25 mL of PBS pH 7.4 (add 25 mL of PBS, mix well with the pipet and wait until the beads are settled down then discard the supernatant and repeat) and one time with 25 mL of DMEM medium.
3. Cover the beads in the 50-mL tube with 10mL of medium 199 supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-Glutamine, 0.4% of Endothelial Cell Growth Medium Supplement Mix and 25 µL of heparin (5000 IU/mL) and allow equilibration for 10 min before further use.

4. Collecting Cells

1. Remove the cell culture medium from the T175 flask containing PAEC and add 5 mL of PBS pH 7.4.
2. Remove PBS from the T175 flask.
3. Add 5 mL of Trypsin-0.05% EDTA and incubate for 3 - 4 min at 37 °C.
4. Collect the cells by rinsing the flask with 15 mL of cell culture medium and transfer the suspension in a 50-mL tube.
5. Centrifuge cells at 1,200 x g for 8 min at room temperature, remove excess medium and resuspend the pellet in 5 mL of cell culture medium.

5. Seeding Cells into the Stirrer Flask

1. Add 20 mL of cell culture medium to the cell suspension and resuspend.
2. Add 20 mL cell culture medium (w/o cells) into the 500-mL magnetic stirrer flask.
3. Add the cells to the washed microcarrier beads from step 3.3 and mix carefully with a 25-mL serological pipette.
4. Transfer the beads/cell mixture into the magnetic spinner flask.
5. Rinse the 50-mL tube with 10 mL of cell culture medium to collect the remaining cells.
6. Add an additional 85 mL of cell culture medium into the spinner flask and place it into the incubator overnight at 37 °C on a shaker (100 x g, mixing interval: 3 min every 45 min).
7. Add 50 mL of cell culture medium (total volume 200 mL) and continue stirring for additional 24 h at 37 °C on a shaker (100 x g, mixing interval: 3 min every 45 min).
8. Add colorless RPMI medium (supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-Glutamine, 0.4% of Endothelial Cell Growth Medium Supplement Mix and 25 µL of Heparin) until 320 mL of total volume is reached.
9. Replace the medium every 48 h: Remove 100 mL of old medium and add 100 mL of fresh supplemented colorless RPMI.
10. Culture the cells for 5 to 7 days. The time depends on the confluence state of the cell-coated beads.

6. Confluence Verification

1. Collect 200 µL of cell-coated beads using a pipette and transfer them into a polypropylene tube.
2. Wash the beads 3 times with 600 µL of PBS 1x (add PBS, tilt the tube and mix gently to avoid detachment of the cells, wait the beads to settle down, discard the PBS and repeat).
3. Fix the beads for 10 min by adding 200 µL of parapicric acid.
4. Wash 3 times with 600 µL of PBS 1x.
5. Add DAPI diluted in PBS 1x and incubate for 10 min.
6. Transfer the beads on a glass slide and apply a coverslip using glycerol based mounting medium.
7. Visualize the beads under a confocal microscope.

7. Experimental Procedure

1. Remove the cell-coated beads from the magnetic stirrer flask (procedures do not have to be done under sterile conditions) with a 10-mL serological pipette and transfer them into 12 mL round-bottom polypropylene tubes.
2. Let the beads settle down (around 1 - 2 min) and remove excess medium.
3. Add more beads to the tubes until every tube contains exactly 2 mL of beads.
4. Add 5-mL clear RPMI to each tube and mix carefully using a 10-mL serological pipette.
5. Let the beads settle down and remove excess medium.
6. Repeat the washing procedure one more time with RPMI and remove all excess medium.

8. Incubation with Non-anticoagulated Blood

1. Carefully and slowly (using neither jet nor vacutainers) draw blood from a healthy volunteer and collect it in 9 mL neutral polypropylene tubes (no anticoagulant).
2. Slowly transfer 8 mL of blood with a 10-mL serological pipette into each of the polypropylene tubes containing 2 mL of cell-coated beads (the total volume will be 10 mL). Always avoid rough handling of blood or beads to avoid premature EC activation. The procedure takes 1 - 2 min.
3. Carefully tilt the blood/bead mixture to ensure equal mixing and seal the cap with paraffin film.
4. Place the tubes on a horizontal tilting table (with gentle tilting settings only) inside a 37 °C incubator and record clotting times.
    1. At set time intervals, *e.g.* after 10, 20, 30, 50, 70, 90 min, remove at least 1.5 - 2 mL of blood-bead mixture for serum or plasma analysis.

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NOTE: for 6 time points we suggest having more than 3 replicates within one group of cells, as the blood sampling will be done in different tubes.

2. For collection of serum, leave the blood to coagulate. To collect the plasma, add EDTA or citrate to 2 mL tubes before adding blood samples.
3. Store the tubes on ice, centrifuge at 2,500 x g for 10 min at 4 °C and store serum/plasma at 80 °C until use.
   NOTE: Details on materials are provided in **Table of Materials**

Representative Results

After 7 - 10 days of culture in the spinner flask (**Figure 1**) the cells were confluent, covering the whole surface of the microcarrier beads (**Figure 2**). Verification of the confluency state is an important step because a non-confluent monolayer of EC on the microbeads will lead to a marked decrease in the clotting time, given the microcarrier beads' surface is strongly pro-coagulant (clotting time: 4 ± 1 min) (**Figure 3**).

Another important point which needs attention is the speed of the tilting plate. A high tilting speed will enhance blood clotting. A prolongation of the clotting time could be observed if a monolayer of EC was present on the surface of the microcarrier beads. The use of GalTKO/hCD46/hTBM transgenic PAEC showed a significant increase in the clotting time compared to WT PAEC (**Figure 3**). The absence of the Gal-α-1,3-Gal xenoantigen on the PAEC (GalTKO) showed an increase in clotting time (25 ± 8 min for PAEC WT and 68 ± 30 min for PAEC GalTKO). Another strong increase in clotting time was observed when PAEC GalTKO/hCD46/hTBM were present on microcarrier beads (205 ± 32 min), which suggests a successful modulation of both the complement (hCD46) and coagulation systems (hTBM). The end of the experiment is defined when a visible clot is formed.The variability within samples of the same of group is due both to inter assay-variability and the blood donor. Every experiment had 3 replicates and each time a different blood donor was used to increase the reliability of the data. For each donor, a blood analysis (platelet count, WBC, RBC, HCT and other parameters) was performed by external healthcare laboratories. The results shown in **Figure 3** are obtained from different experiments performed with different blood donors.

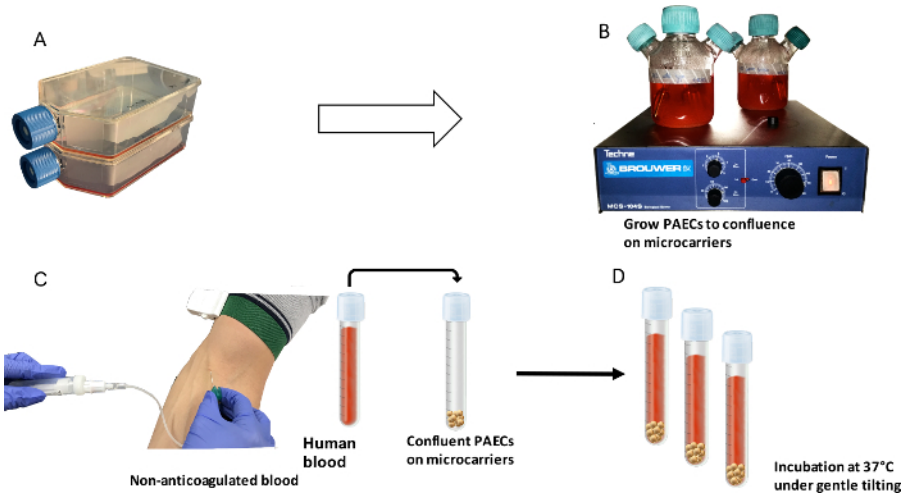

Figure 1: Schematic Representation of the Microcarrier-based Assay. (A) EC are expanded in T175 flasks and **(B)** transferred into spinner flasks together with collagen-coated microcarrier beads. **(C)** Fresh non-anticoagulated blood is collected from a healthy volunteer, **(D)** mixed with EC-coated microcarrier beads, and incubated at 37 °C. The phrase "non-anticoagulated blood" means that the blood has not been treated with any anticoagulant. Please click here to view a larger version of this figure.

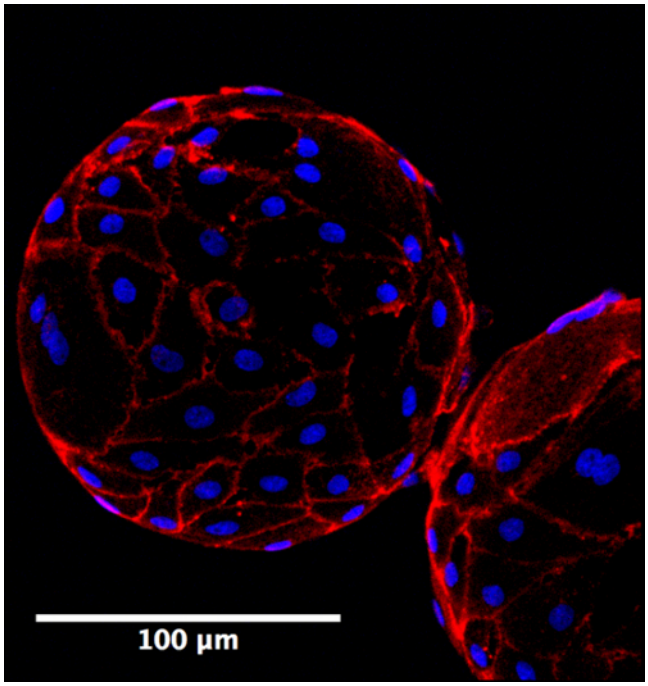

Figure 2: Immunofluorescence Staining on EC-coated Microcarrier Beads. Microcarrier beads were retrieved and stained for CD31 (PECAM-1) to assess the confluency. The nuclei were stained in blue (DAPI) and CD31 was stained in red (Cy3). Scale bar: 100 µm. Please click here to view a larger version of this figure.

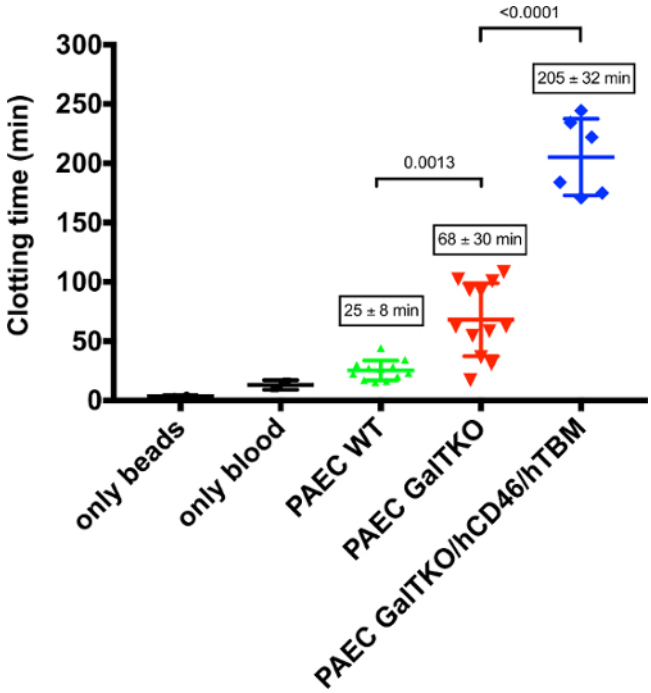

Figure 3: Clotting Times of Different EC. Clotting times were determined visually and the end of the experiment was defined when a visible clot was observed. A strong procoagulant effect was observed when non-EC-coated microcarrier beads were exposed to whole non-anticoagulated human blood. The absence of the Gal-α-1,3-Gal xenoantigen on the PAEC (GalTKO) showed an increase in clotting time (25 ± 8 min for PAEC WT and 68 ± 30 min for PAEC GalTKO). A further increase in clotting time was observed when PAEC GalTKO/hCD46/hTBM were present on microcarrier beads, which suggests a successful modulation of both the complement (hCD46) and coagulation systems (hTBM). Data are shown as mean ± standard deviation. Statistical analysis has been done using ANOVA for multiple comparisons with Bonferroni correction. Please click here to view a larger version of this figure.

Discussion

The model presented here is suitable for coagulation related studies allowing the analysis of different aspects of coagulation and its interaction with EC.[8] In xenotransplantation research, it is a useful system to test the anticoagulant properties of different genetically modified porcine ECs after incubation with human blood [9].

The most critical steps of the protocol are those ensuring complete cell coverage (confluency) of the microbeads before starting the experiment and those pertaining to the careful collection and handling of the non-anticoagulated whole blood to avoid premature platelet activation due to high shear stress, which may occur if vacutainers are used to collect the blood.[10] Nevertheless, this system has some limitations, including the absence of a recirculating closed system and the absence of physiological shear stress which would better represent the *in vivo* conditions.

Despite these limitations, the described model offers significant advantages over currently existing models. Due to the use of microcarrier beads, the EC surface to blood volume ratio is increased, allowing the establishment of the anti-coagulant and anti-inflammatory environment and use of non-anticoagulated whole blood. In current flat-bed cell culture models, this is not possible and mechanisms which involve blood clotting due to EC activation therefore often require the use of animal experimentation. In part, this can be avoided using the described microcarrier bead model.

Furthermore, this system allows for a broad spectrum of applications. Its versatility resides in the possibility of testing different drugs or compounds which are not only related to (xeno-) transplantation but also to human diseases. The effects of the drugs on the endothelium and on the coagulation system can be easily assessed by immunofluorescence, ELISA and multiplex suspension array analysis. This was previously done in a study on the effect of transgenic expression of human thrombomodulin in a xenotransplantation setting.[11]

A possible modification of the described method could be the collection of non-anticoagulated whole blood directly into tubes which are filled previously with cell-coated beads in order to reduce air-contact and blood activation by using the pipette. The use of human aortic endothelial cells (HAEC) may be an interesting control and is already incorporated into future plans. Argon topping of the tubes might be used to reduce the contact of non-anticoagulated blood with air, which is known to lead to contact activation of the clotting cascade. If the blood is drawn too quickly, for example by using vacuumed tubes with rubber stopcocks and introducing blood into the tube in a fine jet, then platelets become activated and coagulation will occur sooner. To avoid platelet activation, use large diameter hypodermic needles (21G - 16G).

Disclosures

The authors have nothing to disclose.

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