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Visualization of adherent cell monolayers by cryo-electron microscopy: A snapshot of endothelial adherens junctions

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ABSTRACT

Cryo-electron microscopy (cryo-EM) allows the visualization of the cell architecture in its native state. We developed a robust solution to adapt cryo-electron microscopy of vitreous sections (CEMOVIS) to a monolayer of adherent cells using a functionalized polyacrylamide hydrogel growing substrate. We applied this method to reconstitute an endothelial cell monolayer to visualize the morphology of adherens junctions (AJs) which regulate permeability and integrity of the vascular barrier. The fine morphology and ultrastructure of AJs from cultured primary human coronary artery endothelial cells (HCAECs) were analyzed in their native state by using CEMOVIS. Doxycycline and sphingosine-1-phosphate (S1P) are known as efficient regulators of endothelial permeability. Doxycycline and S1P treatments both led to a drastic morphological switch from very uneven to standardized 14–17 nm wide AJs over several microns indicative of a better membrane tethering. Repetitive structures were occasionally noticed within the AJ cleft reflecting a local improved structural organization of VE-cadherin molecules. The ultrastructural stabilization of AJs observed upon treatment likely indicates a better adhesion and thus provides structural clues on the mechanism by which these treatments improve the endothelial barrier function. This method was also successfully extended to a thick epithelial barrier model. We expect our strategy to extend the reliable application of CEMOVIS to virtually any adherent cultured cell systems.

1. Introduction

Cryo-electron microscopy (cryo-EM) allows the visualization of unstained isolated macromolecules or biological assemblies in their native state, frozen in a thin layer of amorphous ice avoiding the artifacts that are known for conventional electron microscopy specimen preparation (chemical fixation, dehydration). High pressure freezing (HPF) is currently the most efficient and popular cryoimmobilization technique for tissues and cultured or isolated cells specimens. HPF flat specimen carriers are used for tissues while copper tubes are more suitable for cell suspensions (Dubochet, 2007). Subsequently, the thickness of vitreous biological samples is most commonly reduced by cryosectioning using the so-called CEMOVIS method (Al-Amoudi et al., 2004; Hsieh et al., 2006). Cryo-EM observation of adherent cells is facing an additional constraint due to the presence of cell growing supports. Sapphire discs, polyethylene and Aclar® copolymer films are growth supports compatible with cell vitrification when fitted into HPF flat carriers (Brown et al., 2012, 2009; Jiménez et al., 2010; McDonald, 2009; Spiegelhalter et al., 2010). However their cryosectioning or detachment from the vitrified sample generally cannot be achieved without damaging the sample. Thus, adherent cells grown on the aforementioned supports had so far to be processed by freeze substitution and plastic embedding and could never be used for CEMOVIS. An alternative method consists in growing cells directly on thin carbon coated electron microscopy grids, followed by plunge freezing in liquid ethane. Whereas peripheral parts of some cell types are thin enough to be correctly vitrified, cell bodies are usually too thick to be properly cryo-fixed or examined through cryo-EM. To overcome these drawbacks, another option is to grow cells on dextran microbeads that can be filled into copper tubes for HPF and cryosectioning (Hagen and Grünewald, 2008). Although the method has proved successful, a main pitfall of microbeads

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come from their inherent spherical geometry that make the monitoring of important cell culture parameters such as cell proliferation and confluence more challenging. Cell culture conditions also have to be adapted to culture in suspension, which is not straightforward with every cell type. Polyacrylamide (PAA) hydrogels have recently gained growing interest in the field of biomedical engineering thanks to their inherent biological inertness and compatibility as well as the ease in tuning their physicochemical properties. By varying their acrylamide or cross-linker concentrations, PAA hydrogels functionalized with extracellular matrix proteins (ECM) have been introduced as cell culture substrates to study the effect of substrate stiffness on morphology, spreading and motility of adherent cells (Kandow et al., 2007; Pelham and Wang, 1997; Tse and Engler, 2010; Wang and Pelham, 1998; Yeung et al., 2005).

In this report, we propose a method using PAA hydrogel as growth support for adherent cell monolayers, amenable to cryo-EM structural studies. We applied this method to reconstitute an endothelial cell monolayer to get access to the morphology of adherens junctions (AJs). In vascular endothelium (VE), AJs mainly composed of the integral protein VE-cadherin play important roles in the maintenance of vascular integrity. Increased vascular permeability has been observed in diseases e.g. acute inflammation, tumor angiogenesis, and atherosclerosis and is mainly mediated by histamine, endothelial growth factor (VEGF) and tumor necrosis factor (TNFα) (Andriopoulou et al., 1999; Chen et al., 2012; Esser et al., 1998; Gavard and Gutkind, 2006; Petreaca et al., 2007; Sidibé et al., 2012; Vilgrain et al., 2013; Wallez et al., 2007). Little is known about AJ structure and VE-cadherin organization, mainly because of the aforementioned technical difficulties to observe AJ at nanometer level under native conditions. Therefore endothelial cells were grown on flat specimen carriers coated with ECM protein functionalized PAA hydrogel and submitted to HPF. Selection of regions of interest (ROI) was achieved by coupling fluorescence imaging to cryo-ultramicrotomy. We describe the morphology of AJs in their native state using cryo-EM and provide new insights into their morphological modifications upon treatment with sphingosine-1-phosphate (S1P) or doxycycline that are efficient regulators of endothelium permeability (Adamson et al., 2010; Fainaru et al., 2008; Garcia et al., 2001; McVerry et al., 2004; Sun et al., 2009).

2. Materials and methods

2.1. Material and chemical

3 mm type A (cavity 200 μm) and type B (flat surface) copper gold-plated carriers were purchased from Leica. Unless otherwise stated, all chemicals and biological compounds were from Sigma.

2.2. Hydrogel coated carrier preparation

Type A carriers were cleaned for 2 h in a mixture of sulfuric acid and hydrogen peroxide (piranha solution), then thoroughly rinsed with water and air dried before use. To prepare PAA hydrogel, a solution of 250 μL of 20% (w/v) acrylamide/bis-acrylamide (37.5:1) kept on ice for 10 min was mixed with 4 μL of 10% ammonium persulfate and 0.3 μL N,N,N′,N′-tetramethylethylenediamine (TEMED). Then 1 μL of this prepolymer solution was poured into the 200 μm cavity of type A carrier. During the 20 min polymerization time, a stamping tool was used to adjust hydrogel thickness to about 100 μm (see Fig. S1). For surface functionalization, hydrogel covered with 300 μL of 50 mM sulfosuccinimidyl-6-(4′-azido-2′-nitrophenylamino)hexanoate (Sulfo-SANPAH; Pierce) in buffer (50 mM Hepes, pH 8.5) was exposed to 365 nm UV light (Uvitec model LF-204.LS) at a distance of 6 cm for 15 min as adapted from Pelham and Wang (1997). Sulfo-SANPAH solution was then exchanged and photoactivation procedure repeated once. Photoactivated hydrogels were washed 2 times in buffer and coated for 2 h with 10 μg/mL human fibronectin dissolved in the same buffer containing 0.2% (v/v) acetic acid. After brief washing, type A carriers were ready for primary human coronary artery endothelial cell (HCAEC) culture. An alternative method for protein coating was preferred for caco-2 intestinal epithelial cells. Collagen I (rat tail; Life technologies) was directly cross-linked to acrylamide avoiding Sulfo-SANPAH treatment. Briefly, 125 μL of 40% acrylamide/bis-acrylamide (37.5:1) was mixed with 110 μL of 300 μg/mL precooled collagen and 10 μL of 250 μg/mL heterobifunctional cross-linker acrylic acid N-hydroxysuccinimide ester (AA-NHS) prepared in precooled PBS pH 7.2 as adapted from Schnaar et al. (1978). The mixture maintained at 4 °C was incubated for 10 min before polymerization initiation as described above. In bulk collagen cross-linked hydrogel was then ready for caco-2 cell culture.

2.3. Cell culture

Hydrogel filled carriers were transferred to 4-well culture dishes prefilled with culture medium and allowed to equilibrate for 30 min in a humidified atmosphere with 5% CO_2 at 37 °C before seeding cells. HCAEC purchased from Lonza were used at passages 3–4 and cultivated in EGM-2 MV complete medium containing 5% fetal bovine serum (FBS) and growth supplements (Lonza). Cells were seeded near confluence at 50,000 cells/cm^2 and grown for 2 days. Caco-2 cells (Hidalgo et al., 1989) (gift from Dr. A. Hadj Sassi) were grown in DMEM containing 4 mM L-glutamine, 4.5 g/L D-glucose, 1 mM sodium pyruvate and supplemented with 10% decomplemented FBS, 1% non-essential amino acids and 1% penicillin/streptomycin (Gibco, Life technologies). Cells were seeded at 50,000 cells/cm^2 and allowed to differentiate for 30 days with daily medium changes after the first 48 h. Treatment with S1P was performed for 1 h at a 1 μM final concentration from a methanol 1 mM stock solution. The carrier solution had no effect on the AJ morphology. Treatment with doxycycline was applied for 16 h at a 30 μM concentration from a freshly prepared aqueous stock solution. For targeted ultramicrotomy, calcein acetoxymethyl ester (calcein-AM; AAT bioquest) was added at 2 μM to the medium for 20 min just prior to HPF.

2.4. High pressure freezing, cryosectioning and cryo-electron microscopy

Carrier was removed from culture dish and culture medium blotted without drying. 2 μL of a 20% dextran solution (40 kDa) prepared in serum-free medium was layered onto cells and homogenized by up and down pipetting while avoiding insertion of air bubbles. Dextran volume was adjusted to form a slightly convex surface. Type B carrier was fitted on top of Type A, flat side coated with lecithin facing dextran. The assembly fitted in the plastic holder was vitrified with an HPM 100 apparatus (Leica) and stored as such in liquid nitrogen. The assembly was punched out from its plastic holder and opened with tweezers within the cryochamber (FC6) of the ultramicrotome (UC6; Leica) set at −140 °C. The type A sample carrier was then loosely clamped to the microtome arm at -140 °C using an AFM specimen holder (Leica) preloaded with some cryoglue (Isopropanol/Ethanol 90/10 (vol/vol)). Firm attachment was further provided by lowering temperature to −150 °C making cryoglue to set hard. Pyramid was trimmed using a diamond trimming knife (cryo-trim 45° or 20°, Diatome). For targeted microtomy, fluorescence visualization of the cells was done using an LED based lab-made fluorescence setup fitted around the standard microtome stereomicroscope (Fig. S2).