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The use of mild trypsinization conditions in the detachment of endothelial cells to promote subsequent endothelialization on synthetic surfaces

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Abstract

A necessary condition for endothelialization of small diameter grafts is rapid and firm adhesion of endothelial cells upon exposure to flow. To retain integrins on the cell surface, we assessed the effects of trypsin concentration, the duration of trypsin incubation, and trypsin neutralization methods on endothelial cell adhesion. Human umbilical vein endothelial cells which were detached using 0.025% trypsin for 5 min and seeded onto glass pretreated with fibronectin had close to 100% cell retention when shear stresses as high as 200 dyn/cm^2 were applied for 2 min. An equivalent level of cell retention was observed on fibronectin coated Teflon-AF^{TM} for shear stresses up to 60 dyn/cm^2 applied for 4 h. Using 0.025% trypsin, initial cell spreading and cell surface $\alpha_5\beta_1$ integrins were increased relative to cells treated with 0.5% trypsin. After 1 h of attachment, focal adhesions formed when low trypsin concentrations were used but were less evident with high trypsin concentrations. These results showed that low trypsin concentrations produced faster spreading, a higher number of intact integrins, and rapid focal adhesion formation.

Keywords: Cell adhesion; Cell culture; Cell spreading; Endothelial cell; Integrin; Shear

1. Introduction

There is considerable interest in promoting rapid and firm adhesion of endothelial cells (ECs) for a number of clinical applications, including seeding of vascular grafts and vascular tissue engineering [1]. ECs perform many vital functions: regulate platelet activation, adhesion, and aggregation; limit leukocyte adhesion; regulate smooth muscle cell migration and proliferation; and control blood flow and vessel tone [2–5].

EC adhesion to materials is mediated by adsorbed cell adhesion proteins, primarily fibronectin and/or vitronectin [6,7]. Cell adhesion proteins bind to cell surface integrins, a family of heterodimeric transmembrane proteins consisting of α and β subunits. Integrins $\alpha_5\beta_1$ and $\alpha_V\beta_3$ both play a major role in EC adhesion. Binding to extracellular matrix proteins activates integrins to associate with the actin cytoskeleton and eventually leads to integrin clustering into focal adhesions [8]. The formation of focal adhesions, which contain actin-associated proteins such as talin, vinculin, paxillin, and α-actinin, strengthens the adhesion of cells to the substrate [9].

Critical shear stress experiments are a common method used to quantify cell's adhesion strength to specific substrates. Prior to seeding a synthetic surface, ECs are usually treated with the proteolytic enzyme, trypsin, which cleaves proteins at the carboxyl side of the basic amino acids lysine and arginine, in order to remove cells from their culture substrate and hence be used for further applications [10]. Both $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins are sensitive to trypsin [11].

Of those studies that do report the trypsin concentrations and incubation times to detach cells, the trypsin concentrations to detach cells range from 0.05% to 0.5% [13–24] and incubation times range from 5 to 10 min

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[12–14] at either room (25 °C) or physiological temperature (37 °C). Taking the steps to neutralize the trypsin may also be critical for later cell attachment and spreading. The cell suspension is either centrifuged directly and resuspended in culture medium or a neutralizing solution containing serum or calcium ions is added to inhibit the trypsin before centrifugation.

While previous articles have reported relatively low critical shear stress values of 12–37 dyn/cm^2 after 15 min to 1 h of cell attachment [5,6,9,14–17], no direct assessment is reported in the literature on the effect of trypsin on the critical shear stress needed to detach 50% of the cells from the surface.

In a clinical setting there is limited time for either cultured or freshly isolated ECs to strongly attach and spread on graft surfaces. Therefore, it is important to develop methods to promote rapid and strong cell adhesion. We have optimized in vitro conditions to yield very high adhesion strength after short periods of attachment to glass and Teflon-AF^{TM}. Differences in cell adhesion were characterized by the effect of trypsin on initial cell attachment, cell spreading, the number of intact integrins ($\alpha_5\beta_1$ and $\alpha_V\beta_3$) present, and the localization of vinculin at 1 h post attachment.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) (Cambrex BioScience Inc., Walkersville, MD) were grown to confluence in T-25 or T-75 polystyrene flasks (Becton Dickinson and Company, Franklin Lakes, NJ) with endothelial basal media (EBM, Cambrex) supplemented with EGM SingleQuots (Cambrex) and 1X antibiotic/ antimycotics solution (Gibco, Carlsbad, CA). Cells were cultured in a tissue culture incubator with 95% air/5% CO_2 at 37 °C. HUVECs were used at passage 3–6 for all experiments.

After rinsing HUVECs with Dulbecco's phosphate buffered saline solution (DPBS, Gibco) without Ca^{2+} and Mg^{2+}, the conditions in Table 1 were examined to determine the effect of trypsin (Gibco) concentration, trypsin incubation time, and trypsin neutralizing solution (TNS) (Cambrex). Once the cells were trypsinized and the appropriate neutralization solution was added, the cell suspension was immediately centrifuged, resuspended in either DPBS or serum free media (serum free media consisted of EBM and EGM SingleQuots minus the fetal bovine serum), and then used in specific experiments. For each of the trypsin concentrations, cell detachment was complete at the end of the incubation period.

2.2. Substrates

Cell adhesion and spreading studies were performed using either standard $1'' \times 3'' \times 1$ mm soda lime glass slides (Gold Seal, Portsmouth, NH) or Teflon-AF^{TM} (DuPont, Wilmington, DE) films spun-cast onto standard glass microscope slides. Prior to use, the glass slides were cleaned by sonication with 2% PCC-54 detergent cleaning solution (Pierce, Rockford, IL) and a 1:1 mixture of MeOH:HCl as previously described and the Teflon-AF^{TM} was spun-cast onto the clean microscope slides also as previously described [1]. All glass slides were pretreated with 1 ml of DPBS containing both 10 μg/ml fibronectin (Sigma) and 200 μl of 2 mg/ml bovine serum albumin (BSA, Sigma) for 1 h at 37 °C, unless noted otherwise. Teflon-AF^{TM} coated slides were incubated with 1 ml of 10 μg/ml fibronectin in DPBS for 1 h at 37 °C.

2.3. Cell adhesion strength and cell retention

Cell detachment conditions, summarized in Table 1, were used to determine the effect of trypsin conditions on initial adhesion and retention. ECs were resuspended in DPBS and attached to the surfaces for 5 min at room temperature. Images of adherent cells were obtained at 5 specific positions at 10 × magnification using phase contrast microscopy (Nikon Diaphot, Tokyo, Japan). Next, slides were gently rinsed three times by slowly removing slides from DPBS at an angle. We had previously determined that three rinses were sufficient to remove non-specifically bound cells from the slides treated with BSA only. A second set of images was taken at the same five positions post dip rinse and the percent of adherent cells per field after rinsing was determined. Three experiments were performed for each of the conditions listed in Table 1.

After rinsing, the slide was placed in a variable height flow chamber which produces a range of shear stresses [12]. A set of 5 images was taken at 5 different channel heights along the chamber. Steady laminar flow was applied for 2 min through the use of a dual syringe pump. The total elapsed time from initial cell attachment to the onset of flow was typically 20 min. The flow media consisted of DPBS with 1.9% dextran (molecular weight, 2×10^6; Sigma) to produce a viscosity of 1.9 centipose, which exhibited Newtonian fluid behavior. The flow was set at a constant rate and the shear stress was computed by

$$\tau = \frac{6\mu Q}{wH(x)^2},$$

where μ is the media viscosity, w is the width of the flow channel, Q is the volumetric flow rate (ml/min), and $H(x)$ is the height of the flow chamber as a function of position along the microscope slide [12]. Shear stresses typically ranged from 20 to 100 dyn/cm^2. To produce shear stresses as high as 200 dyn/cm^2, the flow rate was increased and viscosity was raised to 2.5 centipose (2.5% dextran), which still exhibited Newtonian fluid behavior. After flow exposure, images were taken at exactly the same positions as the pre-flow images, and the cells were counted in both images to determine the number of cells that remained adherent post-flow. Results were normalized to the number of cells present prior to rinsing. Experiments were performed in triplicate.

2.4. EC adhesion to glass and Teflon-AF^{TM} exposed to flow

For 4 h flow experiments, HUVECs were detached from flasks using low trypsin concentrations (0.025% trypsin for 5 min at 37 °C and neutralizing with TNS), resuspended in serum-free media, and seeded onto glass or Teflon-AF^{TM} slides for 1 h at 37 °C prior to flow. Before the 1 h incubation time was over, the slides were assembled in a variable height flow chamber and images were taken at 5 positions at 5 different channel heights. The flow chamber was then connected to a circular flow loop [12] and flow was applied for 4 h using EBM media with 2% fetal bovine serum (no additional growth factors were added to the media). After the 4-h flow, images were taken again at the same exact locations as the pre-flow images. Cells were counted in the pre- and post-flow images in order to determine the percentage of cells that remained adherent post flow at the varying shear stress values. The 4-h flow experiments on glass and Teflon-AF^{TM} were each performed three times.

2.5. Cell spreading

ECs were resuspended in serum free media after detachment using the conditions listed in Table 1 and seeded onto clean glass slides. One hour after seeding, the media was aspirated and replaced with media containing 2% fetal bovine serum (we found that the cells would not survive for more than a few hours without serum being present, data not shown). For each of the 6 conditions listed, images were taken using a phase contrast