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In Vitro Study Of Cytokine-mediated Activation of Endothelial Cell Permeability Using Falcon® Cell Culture Permeable Supports

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Introduction

Cytokine-mediated activation of vascular endothelial cells is an important element in the *in vivo* expression of delayed hypersensitivity[1]. The characteristic delayedonset erythema and induration of delayed hypersensitivity inflammatory reactions are clear evidence of endothelial cell activation and increased macromolecular permeability. Although some aspects of cytokine-mediated endothelial cell activation have been elucidated by *in vivo* histological and physiological studies, further analysis of their mechanism requires use of appropriate *in vitro* model systems. Endothelial cell monolayers cultured on filter membranes bonded to plastic cylinders that can be inserted into tissue culture plates have been used to analyze cytokine-mediated changes in endothelial cell function[2]. Because cultured endothelial cells are easily dislodged from the membrane during routine handling, confirmation of monolayer continuity is critical to validating results of any permeability studies with these systems. Unfortunately, the filter membranes previously available are translucent and routine visualization of the living monolayers by light microscopy to confirm confluence and continuity has been difficult. To overcome the problem of visualizing endothelial cell cultures by light microscopy, we developed a model based on Falcon 3.0 µm Cell Culture Permeable Supports. These permeable supports incorporate a transparent, optically flat polyethylene terephthalate (PET) membrane. Monolayer cultures of human umbilical vein endothelial cells (HUVEC) grown on these permeable supports have been used in fluorometric assay to examine activation of permeability by recombinant human interleukin-1 (rhuIL-1). The optical clarity to the PET membrane has enabled us to use phase-contrast light microscopy to confirm the confluence and continuity of HUVEC monolayers during the permeability assays, and to thus avoid using cultures with damaged monolayers. Our results indicate that Falcon Cell Culture Permeable Supports with PET membranes provide an excellent experimental system for studying cytokine-activated changes in endothelial cell permeability.

Materials and Methods

Cells and Reagents: HUVEC were obtained from isolated umbilical veins by a standard method and grown in Medium 199 supplemented with 100 µg/ml heparin, 3 µg/ml thymidine, 10 ng/ml bovine ECGF, antibiotics, 20 mM HEPES and 20% FBS (complete medium)[3]. HUVEC were used for permeability assays between passages 2 and 4. Collagen type I and rhuIL-1b were obtained from Corning Life Sciences (Tewksbury, MA). FITC-lactalbumin was obtained from Molecular Probes (Eugene, OR). FITC-bovine serum albumin (FITC-BSA) was otained from Sigma Chemical Co. (St. Louis, MO). Assay medium contained RPMI-1640 supplemented with 10 mM HEPES, antibiotics and 0.5% BSA. Media was routinely endotoxin-tested. Endotoxin contamination of materials used for HUVEC culture was < 20 pg/ml.

Permeability Assay:

(1) All steps were carried out using sterile technique in a laminar flow hood. Care was taken when changing the medium to avoid letting the membrane dry out at any time once it had been wetted.

(2) Falcon Cell Culture Permeable Supports (3.0 µm) for use with 24-well tissue culture plates were coated with 70 µg/ml type I collagen in 20 mM acetic acid for 1 hour at 23°C. Permeable supports were then washed with HEPES buffered saline (137 mM NaCl-4 mM KCI-6 mM glucose-20 mM HEPES, pH 7.45) to remove excess protein. Complete medium was added to both permeable support and well and the membrane equilibrated for 3 hours at 37°C in 5% CO_2-air.

(3) HUVEC were trypsinized from tissue culture flasks, washed 3 times with complete medium, and seeded on permeable supports at 2 x 10^5 cells/permeable support. Seeded permeable supports were incubated for 48 hours at 37°C in 5% CO_2-air. At the end of incubation and before adding IL-1b, cultures were examined under phase-contrast microscopy, and those with damaged monolayers were discarded.

(4) Recombinant huIL-1b was dissolved in complete medium and added to both sides of HUVEC monolayer, i.e., to both permeable support and lower well. (The stimulus can, of course, be added only to the permeable support or only to the lower well, if desired.) Special care was taken to ensure that the pipet tip did not damage the cell monlayer in this and subsequent steps when changing medium. Permeable supports with HUVEC monolayers were incubated with IL-1b for 18 hours at 37°C in 5% CO_2-air. At the end of incubation with IL-1b, cultures were examined under phase-contrast microscopy, and those with any damage of the cell monolayer were discarded.

(5) To detect changes in monolayer permeability, 750 µl assay medium was added to each lower well and 150 µl assay medium containing 40 µg/ml FITC-lactalbumin or 100 µg/ml FITC-BSA was added to each permeable support. These volumes ensured similar hydrostatic pressure on both sides of the HUVEC monolayer. Incubation was continued for 1 hour at 37°C in 5% CO_2-air.

(6) At the end of incubation, permeable supports were carefully removed, and medium in each lower well throughly mixed. Fluorescence was measured on 150 µl aliquots of medium from lower wells, from FITC-protein assay medium (total fluorescence added to permeable supports), and from assay medium itself (background) using a plate fluorimeter (Fluorescence Concentration Analyzer, IDEXX Laboratories, Westbrook, ME).

(7) Activation of HUVEC monolayer permeability was quantitated as clearance of FITC-lactalbumin from upper well/permeable support to lower well (after subtracting background fluorescence from all values) using the following equation.

$$\text{Clearance (\%)} = \frac{\text{Fluorescence lower well}}{\text{Total fluorescence added upper well}} \times 100$$

(8) After assay of culture permeability, cultures were examined under phase-contrast microscopy for intactness of monolayer. Data from cultures with damaged monolayers was excluded.

RESULTS AND DISCUSSION

HUVEC grew well on the collagen-coated PET membrane, and formed a confluent, intact monolayer 48 hours after they were seeded onto the permeable supports that was easily viewed by phase-contrast microscopy (**Figure 1a**). After incubation with 10 U/ml IL-1b for a further 18 hours, HUVEC monolayers remained viable and confluent and showed no loss of cells from the permeable support membrane (**Figure 1b**). Fewer than 0.2% of cultures had to be discarded because of damaged monolayers. The ease of visualization of HUVEC growing on PET membranes permitted better control for the intactness of the monolayers than would be possible with translucent membranes.

Incubation of HUVEC monolayers with IL-1b caused a significant increase in their permeability to macromolecules (**Figure 2**). Clearance values for the control group of cultures incubated with saline diluent averaged 1% or less. Clearance of fluoresceinated proteins by HUVEC treated with IL-1b was four- to seven fold higher than that of untreated controls, a highly significant difference ($P < 0.01$). As expected because of the difference in molecular weights, clearance of the BSA by activated HUVEC was about half of that of the smaller lactalbumin. In both cases, however, the increase in protein clearance by cytokine-activated HUVEC monolayers was highly significant. The increase in clearance induced by IL-1b in HUVEC measured using FITC-BSA was of the same order of magnitude as that measured using ^{125}I-BSA[4]. Examination of HUVEC by phase-contrast microscopy after completion of assays confirmed the monolayer continuity.

Our results indicate that cytokine activation of HUVEC monolayers cultured on PET membranes can readily be measured using FITC-labeled proteins to provide a rapid, non-radioactive means of determining changes in permeability. Use of FITC labeled proteins has the further advantage of permitting examination of the monolayer for continuity *after* the assay has been completed without worry of radioactive contamination of equipment and personnel.

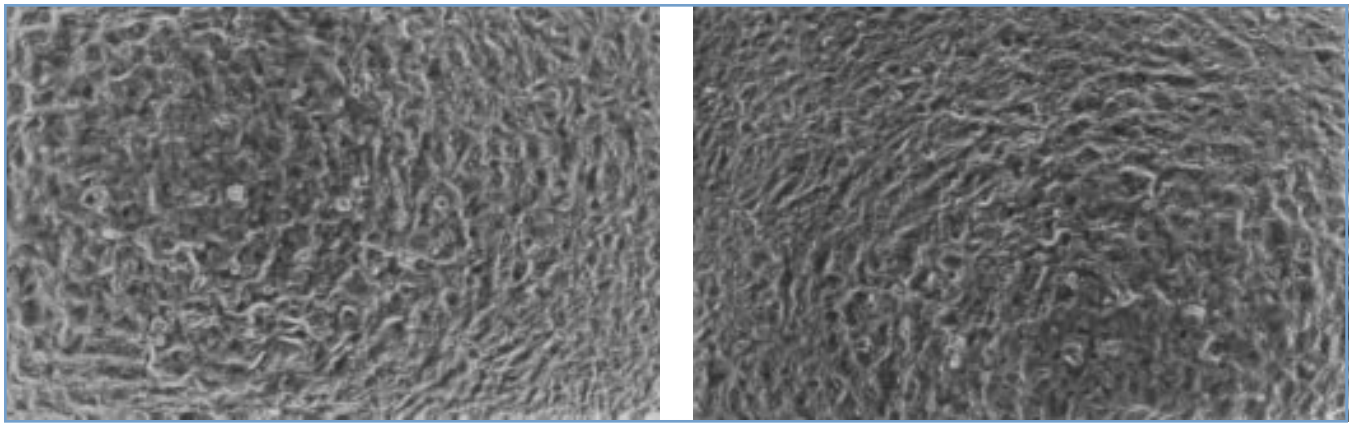

FIGURE 1: Phase-contrast microscopy of HUVEC grown on collagen-coated Falcon® permeable supports with 3.0 µm pores. (left) HUVEC grown in complete medium for 66 hours at 37°C in 5% CO_2-air. (right) HUVEC grown in complete medium for 48 hours at 37°C in 5% CO_2-air and stimulated with IL-1b 10 U/ml for 18 hours. IL-1b stimulation is not associated with any visible damage to HUVEC monolayer. Original magnification = 10x.

HUVEC cultures on collagen-coated membranes are more fragile than the corresponding cultures on a traditional, ridid plastic culture substrate. The cells are easily detached in the course of handling as a result of inadvertent scraping with pipette tips or differences in hydrostatic pressures between the two surfaces of the monolayer. Careful and repeated examinations are therefore necessary to confirm the existence of an intact and undamaged monolayer thorughout the experiment. The transparency of the PET membrane used in the Falcon Cell Culture Permeable Support permits easy and noninvasive control of membrane continuity by light microscopy.

It should also be mentioned using the Falcon Cell Culture Permeable Support for permeability assays is simplified by its convenient design. The plastic extensions on the sides of the permeable support serve as handles for the instruments used to grasp it, and make it easy to manipulate during cultures and assays. As such, it appears particularly well adapted for this use. In sum, Falcon Cell Culture Permeable Supports with PET membranes appear to provide an excellent experimental system for studying cytokine-activated changes in endothelial cell permeability *in vitro*.

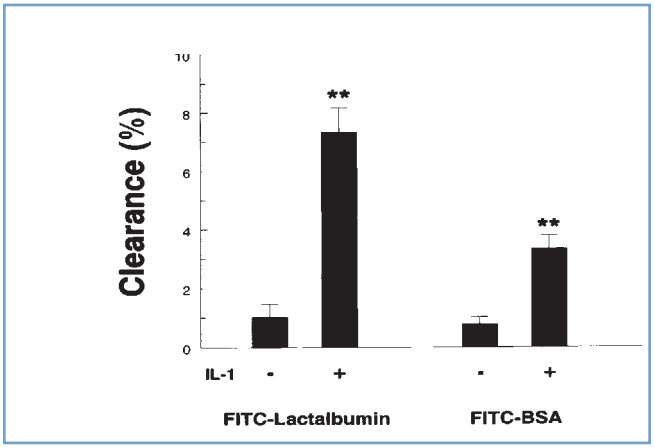

*FIGURE 2: Clearance of fluoresceinated proteins by cytokineactivated HUVEC grown on collagen-coated Falcon permeable supports. Confluent HUVEC monolayers were cultured with (+) or without (-) 10 U/ml rhuIL-1b for 18 hours at 37°C in 5% CO_2-air, and clearance of FITC-lactalbumin and FITC-bovine serum albumin (FITC-BSA) determined fluorometrically. Treatment of HUVEC monolayers with IL-1b significantly increased their permeability to both proteins. **, $p < 0.01$*

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