IN SITU FLUORESCENCE LABELING OF SHEEP LUNG MICROVASCULAR ENDOTHELIUM

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SUMMARY

Endothelial cells are intimately involved in a variety of biological processes such as inflammatory disorders, wound healing, and tumor invasion. The finding of endothelial heterogeneity in various tissues has led to major efforts to isolate and culture microvascular endothelial cells in human and animal tissue. In this report we have used phosphatidyl ethanolamine (PE)-labeled liposomes to fluorescently label the sheep lung microvasculature in situ. Using normotensive perfusion pressure, the PE-labeled liposomes did not extravasate into extravascular lung tissue. Mechanical and enzymatic digestion of the lung tissue demonstrated that the PE-labeled liposomes provided a stable label of the vascular lining cells during ex vivo processing. After digestion, the overwhelming majority of the fluorescent label appeared in cellular aggregates. Approximately 80% of these cells demonstrated an in vitro phenotype consistent with microvascular endothelium. A novel monoclonal antibody selective for sheep endothelial cells was developed to confirm the presence of lung endothelium in the fluorescently labeled cellular aggregates. We conclude that in situ fluorescence labeling of vascular lining cells provides an anatomic marker for relevant vascular lining cells and an opportunity to study these cells in vitro.

Key words: lung; endothelium; microvessels; liposomes; fluorescence; sheep.

INTRODUCTION

The majority of cells that line the small vessels in the lung are microvascular endothelial cells. In contrast to large vessel endothelium, microvascular endothelium seems to be morphologically (Chung-Welch et al., 1989), phenotypically (Belloni and Nicolson, 1988), and antigenically (Kuzu et al., 1992; McCarthy et al., 1991) distinct. Despite the theoretical advantages of using microvascular endothelium for in vitro studies (Scott and Bicknell, 1993), most investigators have used endothelial cells obtained from human umbilical veins or the aorta. Umbilical vein endothelium has the advantage of being a convenient source of endothelium and can be isolated by incubating the vein with proteolytic enzymes (Jaffe et al., 1978). Similarly, aortic endothelium can be isolated by scraping the endothelium with a cotton tip applicator (Schwartz, 1978).

Previous attempts to isolate microvascular endothelium from the lung have identified a number of limitations. The lung contains a significant amount of extracellular matrix composed of both collagen and elastin (Crapo et al., 1983). Also, lung microvascular endothelial cells are morphologically similar to mesothelial cells, making routine light microscopic identification more difficult (Chung-Welch et al., 1989). The most difficult problem in characterizing vascular lining cells is that no single characteristic is sufficient to confirm that a culture is endothelial (Scott and Bicknell, 1993). The traditional markers are the expression of von Willebrand factor (factor VIII-related antigen) and the strong uptake of fluorescently labeled acetylated low density lipoprotein (LDL) (Pitas et al., 1985; Netland et al., 1985). Morphologic descriptions such as a “cobblestone” appearance at confluence are also suggestive of endothelial cells. The problem is that these features are not universal and can even be present with mesothelial cells (Chung-Welch et al., 1989).

In this report, we used phosphatidyl ethanolamine (PE)-labeled liposomes to fluorescently label vascular lining cells in situ. The fluorescence label provided an anatomic and operational definition of vascular lining cells and avoided the selection bias involved in prolonged culture or the assumptions required for selection based on standard surface markers. The fluorescent liposome labels used in this study provided a marker for relevant vascular lining cells. The validity of this approach was confirmed using colony morphology, expression of von Willebrand’s factor, acetylated LDL uptake, and the binding of a novel monoclonal antibody (MAB) selective for sheep endothelium.

MATERIALS AND METHODS

Lung harvest. The lung was removed from a freshly euthanized lamb by extracting the double lung block (Cooper et al., 1987). After cannulation of the pulmonary artery and pulmonary vein using 22-gauge arterial (Harvey) cannulae, the lung was flushed with 1000 ml of 37°C lavage buffer [phosphate buffered saline (PBS), 20 mM HEPES, 1% glucose, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (GIBCO Laboratories, Grand Island, NY)]. The flush routinely removed residual erythrocytes and leukocytes.

Liposome preparation. Fluorescent liposomes were prepared by ethanol injection as previously described (Furlong et al., 1992). Briefly, the liposomes were produced by combining commercially available 2 mg t-a-
phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Avanti, Alabaster, AL) with 3 mg l-a-phosphatidylcholine, and 20 mg/ml chloroform (Avanti) in a round-bottom glass tube. The chloroform was extracted with 100% nitrogen gas. The dried lipid was resuspended in 100 µl of ethanol, and 35 µl of the resulting solution was injected (through a 26-gauge needle on a glass tuberculin syringe) into 10 ml of perfusion buffer. The syringe containing the PE-labeled liposome was raised to the height of 40 cm in a 37°C incubator and the lung was perfused with PE-labeled liposomes for 30 min. During the perfusion, the lung was continuously hand ventilated to promote vascular recruitment.

Purification of lung endothelial cells. The visceral pleura of the flushed lung was peeled off from the lung surface and the outer 2 cm of lung was used to optimize the density of microvascular endothelium while minimizing contamination with large-vessel endothelium and smooth muscle. The lung was processed in a modification of a procedure previously described (Abdi et al., 1993). Briefly, the lung was cut into 1-g pieces and minced into 400-µm cubes. The cubes were digested with enzymatic digestion: 80 U/ml trypsin and 0.8 U/ml Pronase (Sigma) for 30 min. The lung was then processed with the following enzymes: 660 U/ml collagenase (type CLS-1, Worthington, Freehold, NJ), 30 kU DNAase (type I, Sigma), and 60 U/ml elastase (Worthington) in balanced salt solution with 3 mM CaCl₂ and 3 mM MgCl₂ for 1 h at 37°C on a rotary shaker. The lung was filtered through a 100-µm mesh screen to remove any undigested fragments of tissue. The enzymes were quenched by the addition of culture medium with 20% fetal bovine serum. The solution was incubated in culture medium for 1 h at 37°C. The cells were washed twice with culture medium containing 4% sheep serum (Sigma) and resuspended in culture medium containing 20% sheep serum.

Cells and culture medium. The cells were grown in Dulbecco’s modified eagle’s medium (DME) With 2000 mg/liter glucose (Sigma), supplemented with 20% heat inactivated sheep serum (Sigma), 25 mM HEPES buffer, and 2 mM L-glutamine. Endothelial growth supplement (Collaborative Research, Bedford, MA) was added to the medium at 30 µg/ml.