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Development of a coculture system and use of confocal laser fluorescent microscopy to study human microvascular endothelial cell and mural cell interaction

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Abstract

In the present study, human myometrial microvascular endothelial cells (HMMEC) were cocultured with human vascular smooth muscle cells (VSMC) labeled with fluorescent dyes to examine their morphological interaction using confocal laser fluorescent microscopy. HMMEC and VSMC labeled with fluorescent green and red dyes, respectively, attached to opposite sides of polyethyleneterephthalate membranes and remained viable for up to 96 h. In defined medium, 5% ± 3% of the VSMC cytoplasmic processes and 71% ± 17% of the HMMEC processes extended completely across the 13-μm thickness of the transmembrane. However, 41% ± 21% of the VSMC projections and 10% ± 3% of the HMMEC processes that traversed the membrane made contact with the opposing cell type. In cocultures incubated with angiopoietin-1 (Ang-1), although the number of VSMC or HMMEC projections was not significantly increased, the number of VSMC extending across the membrane and making contact with HMMEC was increased ($P < 0.05$) to 88% ± 2%. The results of the current study demonstrate that coculture of fluorescent-labeled HMMEC and VSMC on a semipermeable transmembrane coupled with confocal laser fluorescent microscopy provides an in vitro experimental model to study morphological association of microvascular endothelial cells with mural cells. We propose that this system will greatly facilitate study of remodeling of the microvasculature in various organ systems.

Keywords: Angiogenesis; Vascular cell interaction; Angiopoietin

Introduction

Interaction of endothelial and periendothelial, i.e., vascular smooth muscle cells (VSMC) and pericytes, cells is fundamentally important for microvascular maturation and remodeling (Darland and D'Amore, 2001; Hanahan, 1997; Hanahan and Folkman, 1996). Angiopoietin (Ang-1), acting via its Tie-2 receptor, recruits and promotes association of endothelial cells with VSMC to mature and stabilize newly-formed blood vessels (Darland and D'Amore, 2001; Davis et al., 1996; Maisonpierre et al., 1997; Sundberg et al., 2002; Suri et al., 1996; Teichert-Kuliszewska et al., 2001; Vikkula et al., 1996; Yancopoulos et al., 2000). However, the morphological events and molecular mechanisms underlying blood vessel remodeling are incompletely understood.

Coculture of endothelial cells and VSMC across a semipermeable membrane, which is thought to recreate the in situ orientation of VSMC separated from the abluminal surface of the endothelium by a fenestrated basement membrane, has been developed as a model to study the process of microvascular remodeling (Fillinger et al., 1993; Saunders and D'Amore, 1992). However, evaluation of endothelial–mural cell interaction in this coculture system

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has been primarily by electron microscopy (Fillinger et al., 1993), a procedure which results in membrane disruption and permits examination of a very limited number of cells. Although alternative coculture procedures including an under-agarose assay system have been developed (Hirschi et al., 1998, 1999), the latter appears most useful in assessing early stages of vessel formation, including chemoattractant and mitogenic properties of soluble factors produced by endothelial cells and differentiation of mesenchymal cells to smooth muscle-like mural cells. Therefore, as a first step in our laboratories to study the role of steroid hormones and growth factors on microvascular maturation and remodeling in reproductive tissues such as the uterus and placenta, we modified the coculture transmembrane system and used human endothelial cells and VSMC labeled with fluorescent dyes and confocal laser fluorescent microscopy to study their morphological interaction and the role of Ang-1 on this process.

Materials and methods

Endothelial and vascular smooth muscle cells

Cryopreserved human myometrial microvascular endothelial cells (HMMEC) and human VSMC were obtained at passage 3 (Clonetics Inc., Baltimore, MD), seeded in 100-mm cell-culture dishes (200,000 cells/dish) and cultured in a humidified atmosphere (5% CO_2; 37°C) in media (HMMEC in EGM-2MV BulletKit; VSMC in SmGM-2 BulletKit as recommended by the manufacturer, Clonetics). The manufacturer (Clonetics) also provided fetal bovine serum (FBS), hydrocortisone, human fibroblast growth factor (hFGF), epidermal growth factor (hEGF), insulin-like growth factor 1 (IGF-1), ascorbic acid, and antibiotics which were added to the EGM-2MV media and the FBS, hFGF, hEGF, insulin, and antibiotics to the SmGM-2 media. Attached cells were grown for a pre-confluent period of 5–7 days, trypsinized and either cryopreserved or subcultured in 100-mm dishes. Cells from passages 5–7 were used in the studies described below.

Labeling of cells with fluorescent dyes

HMMEC and VSMC were incubated with 5 μM green tracker dye (5-chloromethyl-fluorescein; CellTracker Green CMFDA; Molecular Probes Inc., Eugene, OR) or 5 μM red dye (5- (and 6)-((4-chloromethyl) benzoyl)-amino)-tetramethylrhodamine; CellTracker Orange; CMTMR; Molecular Probes Inc.), respectively, in EBM-2 base media (Clonetics) for 60 min (37°C) in 5% CO_2. Media was then removed and replaced with fresh EBM-2 without tracker dyes and cells were incubated for 24 h. Media containing dye which leached from cells was removed and cells were harvested and re-suspended in EGM-2MV BulletKit media without hydrocortisone or VEGF and supplemented with 4 ng/ml hFGF (Clonetics; EGM-2MV coculture media). Tracker-dye-labeled HMMEC cells (1.5×10^5 cells in 0.9 ml EGM-2MV coculture media) were plated on inserts with a semipermeable polyethylene terephthalate (PET) membrane (13 μm thick, 1.6×10^6 pores/cm^2, Beckton Dickinson Falcon, Inc., Franklin Lakes, NJ) with 0.45- or 1.0-μm-sized pores. Different pore sizes were used to determine the largest pore that would permit cell processes, but not cells, to pass through the membrane. The inserts were placed upside down in a sterile dish containing 3 ml EGM-2MV coculture media and incubated at 37°C in a 5% CO_2 humidified atmosphere for 4 h to allow cells to adhere. Membrane inserts were removed, media and any non-adherent HMMEC discarded and tracker-dye-labeled VSMC cells (1.5×10^5 in 3.0 ml EGM-2MV coculture media) were plated on the top of the membrane inserts. The inserts containing both cell types were then incubated for 6 h at which point VSMC had attached. Coculture experiments were also performed with HMMEC and VSMC that were not prelabeled with CellTracker dyes.

HMMEC–VSMC coculture experiments

Inserts with HMMEC and VSMC, each approximately 70–80% confluent, were removed and placed into 6-well culture plates containing 3 ml fresh EGM-2MV coculture media supplemented without or with 250 ng/ml Ang-1 (Research and Development Systems, Minneapolis, MN) and 5 μg/ml 6× anti-histidine antibody (Research and Development Systems) to ensure biologic activity of recombinant Ang-1 according to the manufacturer's instructions. Following incubation (37°C) for 24 or 48 h, at which point HMMEC and VSMC were essentially 100% confluent, inserts were fixed in 6 ml 95% ethanol for 45 min, washed in Dulbecco's PBS (Gibco, Grand Island, NY), removed and then mounted between two glass cover slips (22 × 30 mm and 24 × 60 mm; Corning, Corning, NY) with Vectashield mounting media (Vector Laboratories, Burlingame, CA), and sealed with black nail polish. Inserts were then examined using a Zeiss 510 confocal laser fluorescent microscope (Zeiss Inc., Heidelberg, Germany) equipped with an image processing workstation (Metamorph; Universal Imaging Corporation, Allentown, PA). In selected experiments, cells were also examined by fluorescent and bright-field microscopy using an Olympus BX50 microscope (Optical Elements Corporation, Melville, NY) equipped with a Spot Slider digital camera and fluorescence filter set (Diagnostic Instruments, Inc., Sterling Heights, MI).

Image analysis and quantification of HMMEC–VSMC interaction

Initial studies to optimize culture conditions and analysis of cells by confocal laser fluorescent microscopy were performed on 8 different HMMEC–VSMC cocultures