

Isolation of Endothelial Cells and Vascular Smooth Muscle Cells from Internal Mammary Artery Tissue

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ABSTRACT

Analyses of vascular smooth muscle cell and endothelial cell function through tissue culture techniques are often employed to investigate the underlying mechanisms regulating cardiovascular disease. As diseases such as diabetes mellitus and chronic kidney disease increase a patient's risk of cardiovascular disease, the development of methods for examining the effects of these diseases on vascular smooth muscle cells and endothelial cells is needed. Commercial sources of endothelial cells and vascular smooth muscle cells generally provide minimal donor information and are in limited supply. This study was designed to determine if vascular smooth muscle cells and endothelial cells could be isolated from human internal mammary arteries obtained from donors undergoing coronary artery bypass graft surgery. As coronary artery bypass graft surgery is a commonly performed procedure, this method would provide a new source for these cells that when combined with the donor's medical history will greatly enhance our studies of the effects of complicating diseases on vascular biology. Internal mammary artery tissue was obtained from patients undergoing coronary artery bypass graft surgery. Through a simple method employing two separate tissue digestions, vascular smooth muscle cells and endothelial cells were isolated and characterized. The isolated vascular smooth muscle cells and endothelial cells exhibited the expected morphology and were able to be passaged for further analysis. The vascular smooth muscle cells exhibited positive staining for α -smooth muscle actin and the endothelial cells exhibited positive staining for CD31. The overall

purity of the isolations was $> 95\%$. This method allows for the isolation of endothelial cells and vascular smooth muscle cells from internal mammary arteries, providing a new tool for investigations into the interplay of vascular diseases and complicating diseases such as diabetes and kidney disease.

INTRODUCTION

Cardiovascular disease (CVD) remains the leading cause of death in the United States (1). It is a complex process beginning with an initial injury to artery often in the form of lipid deposition initiating a chronic inflammatory response (2–8). As this process continues, there is increased lipid deposition, vascular calcification, endothelial dysfunction, and vascular smooth muscle cell (VSMC) migration and proliferation. The result is the formation of an atherosomous plaque that narrows the arterial lumen, restricting blood flow and presenting the risk of plaque rupture.

Two of the cell types that comprise the artery play key roles in the progression of CVD. The endothelial cells (ECs) serve as a protective lining of the artery and release paracrine factors to the underlying VSMCs that maintain vessel function and health (9–12). Under quiescent conditions, the VSMCs exist in a "contractile" state responsible for maintaining vascular tone. Upon injury and endothelial dysfunction, VSMCs switch to a "synthetic" phenotype, migrating to the intimal layer of the artery where they begin to proliferate (13–15). Thus, regulation of proper EC and VSMC function is central to maintaining vessel health, and therapies targeting these cells have proven effective in treating vascular diseases (16,17).

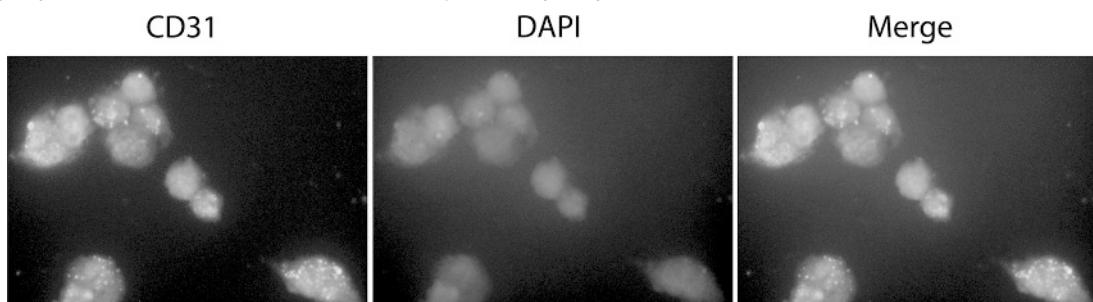
The risk of CVD is greatly increased by the presence of co-morbidities, such as diabetes mellitus or chronic kidney disease. A patient with diabetes but without prior myocardial infarction is at similar risk of cardiovascular death as one with prior myocardial infarction (18). Among dialysis patients, cardiovascular mortality remains the number one cause of death with rates ranging from 10 to 30 times greater than is seen in the general population despite adjustments for

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Figure 1. Representative photomicrograph (63X) demonstrating that the isolated endothelial cells (ECs) stain positive for the EC specific marker CD31. ECs grown on a tissue culture chamber slide were stained with Alexa 647 labeled anti-CD31 antibody (red) and counterstained with the DNA dye DAPI (blue).



other risk factors (19). Furthermore, many of the therapeutic options for treating CVD are less effective in these high risk patient populations (20). These issues highlight the need for laboratory methods that model the interplay of CVD and complicating diseases.

To approach this need, we have developed a method of isolating ECs and VSMCs from portions of the internal mammary artery (IMA) obtained from patients undergoing coronary artery bypass graft (CABG). As part of the tissue acquisition, the relevant medical history is obtained, allowing the stratification of cells into groups of patients with and without complicating diseases. These cells provide us with a powerful model system for exploring the differences in the cellular response to vascular injury brought about by complicating factors such as diabetes and chronic kidney disease.

MATERIALS AND METHODS

Cell Isolation

ECs and VSMCs were isolated using a modification of the murine EC and VSMC isolation method of Kobayashi et al. (21). IMA tissue was obtained from patients undergoing CABG in the Department of Surgery at Ochsner Medical Center – New Orleans. Informed consent was obtained from the patients prior to surgery and this study was conducted with the approval of the Ochsner Health System Institutional Review Board (Protocol 2007.025.A). The tissue was rinsed with Hank's Balanced Salt Solution (HBSS) and clamped at one end. A solution of 2 mg/mL Type I Collagenase (Invitrogen, Carlsbad, CA) in HBSS was injected into the lumen and the tissue was incubated at 37°C for 15 minutes. The clamp was then removed and the lumen flushed with HBSS to collect the ECs. The ECs were plated in a 60 mm tissue culture dish containing human EC growth media (EGM-2, Lonza, Inc., Basel, Switzerland). The adventia was removed, the artery cut lengthwise, and the remaining medial layer cut into 1–2 mm³ pieces. These pieces were digested in fresh 2 mg/mL Type I Collagenase solution

in HBSS for 30 minutes at 37°C. This solution was centrifuged at 1,500 rpm for 10 minutes, and the pelleted VSMCs were collected and seeded in a 60 mm plate containing smooth muscle cell growth medium (SGM-2, Lonza, Inc.). Both isolations were maintained in their respective growth media at 37°C and 5% CO₂.

VSMC Characterization

Cells were seeded on 8-well tissue culture chamber slides (Nalge Nunc, Inc., Rochester, NY) and grown to 50%–70% confluence. The cells were washed twice with cold phosphate buffered saline (pH 7.4, PBS) and then fixed with 10% zinc formalin for 15 minutes at room temperature. Purification of VSMCs was confirmed by positive immunohistological staining for α -smooth muscle actin using a commercial kit (Sigma-Aldrich, Inc., St. Louis, MO) and counterstaining with hematoxylin.

EC Characterization

Purification of ECs was confirmed by positive immunofluorescent staining for the EC surface marker CD31 (PECAM-1). Cells were seeded on 8-well tissue culture chamber slides (Nalge Nunc, Inc.) and grown to 50–70% confluence. The cells were washed twice with cold phosphate buffered saline (pH 7.4, PBS) and then fixed with 10% zinc formalin for 15 minutes at room temperature. CD31 staining was performed using a murine CD31 antibody (MEC 13.3, BD Biosciences, Inc., San Jose, CA) labeled with Alexa 647 by the Zenon IgG Labeling Kit (Invitrogen Carlsbad, CA). ECs were blocked with 10% normal goat serum in PBS for 30 minutes at room temperature and then incubated with the anti-CD31 antibody (1:1000) for 60 minutes. The slide was then washed three times with PBS and fixed again with 10% zinc formalin for 15 minutes at room temperature. The sections were washed again with PBS and mounted using the Prolong Gold antifade mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) nu-

Figure 2. Representative photomicrograph (40X) demonstrating that the isolated vascular smooth muscle cells (VSMCs) exhibit positive staining for α -smooth muscle actin (brown). VSMCs grown on a tissue culture chamber slide were stained for α -smooth muscle actin and counterstained with hematoxylin (blue).



clear stain (Invitrogen, Carlsbad, CA). Immunofluorescence was visualized using an Axiovert 200M Digital Microscopy Workstation and Slidebook Imaging Software (Intelligent Imaging Innovations, Inc., Denver, CO).

RESULTS

Cell Culture and Morphological Analysis

The isolation method described yielded cultures of VSMCs and ECs that were morphologically similar to those of control cultures obtained commercially. Confluent monolayers of VSMCs formed the spindle-shaped pattern common to VSMCs in the contractile state. The ECs exhibited a clear cobblestone morphology characteristic to these cells. Both cell lines grew to confluence and were able to be passaged multiple times without loss of phenotype.

Immunochemical Analysis

To confirm the purity of our isolations, cultures of our isolated cells were stained for specific markers of ECs and VSMCs. Figure 1 demonstrates that the EC cultures exhibited positive staining for CD31. Figure 2 demonstrates the VSMC culture exhibited positive staining for α -smooth muscle actin. Counting of multiple high powered fields confirmed a purity of greater than 95%.

DISCUSSION

This report describes a simple method for isolating ECs and VSMCs from human IMA tissue. The cultures prepared using this method exhibited similar morphology as control cultures and positive staining for appropriate cell markers. The cultures thrived under normal growth conditions and were able to be passaged multiple times, allowing for the use in experimental methods requiring larger cell numbers.

While ECs and VSMCs from various vascular beds are commercially available, the medical history of the donor is generally limited to age, gender, and race. Furthermore, obtaining multiple lots of a specific donor type can be prohibitive due to cost or availability. The isolation of these cells from tissue obtained from patients undergoing CABG provides a new avenue by which to obtain multiple lots with a full medical history. This method will greatly enhance our ability to compare the cellular processes underlying vascular disease in patients with complicating diseases such as chronic kidney disease or diabetes mellitus.

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