Simple, Reliable Isolation, Purification and Cultivation of Murine Skeletal Muscle Microvascular Endothelial Cells

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ABSTRACT

Objectives: Microvascular dysfunction in skeletal muscle is involved in metabolic and vascular diseases. Microvascular endothelial cells (MEC) are poorly characterized in the progression of associated diseases in part due to lack of availability of MEC from various animal models. The objective was to provide a fast, simple, and efficient method to isolate murine MEC derived from skeletal muscle. Methods: Dissected abdominal skeletal muscles from C57BL/6J mice at 8 - 12 weeks of age were enzymatically dissociated. MEC were isolated using a modified two-step Dynabeads™-based purification method. With a combination of Dynabeads™ - *Griffonia simplicifolia* lectin-I and Dynabeads™ - monoclonal antibody against CD31/PECAM-1, MEC were isolated and purified twice followed by cultivation. Results: Isolated and purified cells were viable and cultured. MEC were characterized by using immunofluorescence to identify CD31/PECAM-1, an EC marker, and two specific functional assays, which include a capillary-like tube formation and the uptake of DiI-Ac-LDL. The purity of isolated cell populations from skeletal muscle microvessels, which was assessed by flow cytometry, was 88.02% ± 2.99% ($n = 6$). Conclusions: This method is simple, fast, and highly reproducible for isolating MEC from murine skeletal muscle. The method will enable us to obtain primary cultured MEC from various genetic or diseased murine models, contributing to insightful knowledge of diseases associated with the dysfunction of microvessels.

1. INTRODUCTION

Microvascular endothelial cells (MEC) constitute the inner lining of tiny vessels and play pivotal roles

in blood coagulation, angiogenesis, blood perfusion to tissue, and vascular exchange [1, 2]. Dysfunction of MEC is associated with a wide spectrum of disorders and diseases, such as coronary microvascular disease [3], tumor angiogenesis [4], sepsis, and inflammation [5, 6]. However, the role of microvessels, and particularly that of MEC, in the progression of those diseases remains to be investigated.

Given the heterogenic properties of vascular endothelial cells, physiological and pathophysiological studies using species- and origin-specific cultured MEC are critical for gaining novel insights into molecular mechanisms underlying diseases associated with dysfunction of MEC. Although all vascular EC share some common properties, heterogeneity between microvascular and macrovascular EC have been well documented [7]. Furthermore, a large degree of functional heterogeneity was also found among MEC derived from different tissues [8]. It would be ideal for insightful molecular and functional studies to utilize MEC derived from the same tissue in disease models or genetically engineered mice, and with sex- and age-matched control groups. To that end, the challenge is to obtain large numbers of pure primary cultured MEC.

The purpose of this study is to develop a simple and reliable method for isolation and culture of MEC derived from murine abdominal skeletal muscle. The skeletal muscle is the largest component of body mass in human and the microvessels of skeletal muscles are important constituents of the microcirculation. Microvascular dysfunction of skeletal muscle is involved in the pathogenesis of obesity, diabetes, hypertension, atherosclerosis, and peripheral vascular disease. Endothelial dysfunction in the skeletal muscles associated with these diseases is poorly characterized. Such studies are restricted, in part, by the availability of MEC derived from sex-, age-, and tissue-matched disease models vs. controls.

Although a method using multicolor fluorescent-activated cell sorting (FACS) was reported to improve the purity of isolated cells derived from murine skeletal muscle [9], the sorting is expensive and complicated with technical concerns [10]. Our previous work has demonstrated successful isolation and culture of MEC from rat skeletal muscle. However, it is not simple to directly apply the method used in rats to another rodent species. The specific differences in MEC properties among species, such as proliferation, metabolism, molecular signaling pathways, and responses to various environments, have been reported in stroke [11]. For isolation of mouse MEC, the primary challenges include, but are not limited to, low yield and purity. We proposed to isolate MEC derived from murine skeletal muscle via modification and refinement of our previous method [10, 12] to meet the following criteria: 1) fast and simple preparation; 2) high yield; 3) high purity; and 4) high reproducibility.

Dynabeads™-based two-step purification scheme was employed *Griffonia simplicifolia* lectin-I (GS-I), to recognize the carbohydrate moiety, which is abundantly expressed on the cell surface, followed by monoclonal antibody against CD31/PECAM-1, an endothelial marker. The isolated cells were characterized by using immunofluorescence and functional assays to validate properties of MEC. The purity of isolated cell populations from skeletal muscle microvessels was about 88%, assessed by using flow cytometry.

2. MATERIALS AND METHODS

2.1. Experimental Animals and Reagents

All animal care and experimental protocols on adult male (8 - 12 weeks of age) C57BL/6J mice were conducted in accordance with the "Care of Human Use of Laboratory Animals" under the supervision of Office of Research Administration at Missouri State University. Unless otherwise noted, reagents were purchased from Fisher Scientific (Hampton, NH).

2.2. Isolation and Culture of Skeletal Muscle Microvascular Endothelial Cells

The procedure of MEC isolation was modified from our previous work for rats [12]. The abdominal wall muscles were excised carefully using sterile procedures after the mice were anesthetized with 150 mg/kg ketamine and 7.5 mg/kg xylazine. The excised muscles pooled from four male C57BL/6J mice were cut into ~ 0.5 mm^2 pieces, and then digested in an enzyme solution consisting of dispase (0.12 mg/ml;

Worthington, Lakewood, NJ), trypsin (0.12 mg/ml; Invitrogen, Carlsbad, CA), collagenase type II (0.84 mg/ml; Sigma Aldrich, St. Louis, MO), and bovine serum albumin (BSA, 1.62 mg/ml) in DMEM-F12 (Invitrogen, Carlsbad, CA) at 37°C until the solution became cloudy (40 - 50 min). The cellular suspension was separated from tissue by filtration through sterile gauze and then a cell strainer (40 μm in pore size; BD Falcon, San Jose, CA).

For the first step of the isolation, MEC were isolated using Dynabeads™ coated with GS-I (catalogue: L-2380, Sigma Aldrich, St. Louis, MO) for 10 - 15 min at room temperature and followed by collection of cells that bound to GS-I using a magnet. The cells isolated by the first step were cultured with DMEM-F12 containing 20% fetal bovine serum (FBS; vol/vol %), endothelial cell growth supplement (50 μg/ml), heparin (5 U/ml), antimycotic-antibiotic solution (10 μl/ml), and L-glutamine (0.1 mg/ml).

A second isolation was then performed using Dynabeads™ coated with monoclonal antibody against CD-31/PECAM-1 (BD Biosciences, San Jose, CA) for 30 min at 4°C. The cells were collected using a magnet and cultured with medium containing 10%, instead of 20% FBS. All subcultures (>2 passage) were grown in 10% of FBS medium.

2.3. Identification of Isolated Cells

Immunofluorescence assay. Cells were seeded and grown on a Lab-Tek® II Chamber Slide™. MEC were fixed in 4% (w/v) paraformaldehyde at room temperature (RT) for 10 min and then permeabilized with 0.1% (v/v) Triton-X-100 for 5 min at RT. After blocking with 5% (w/v) IgG-free BSA (Jackson Immunoresearch, West Grove, PA) for 30 min at RT, the cells were incubated overnight with specific mouse monoclonal antibody anti-CD31/PECAM-1 (1:50 dilution; Serotec Immunological Excellence) at 4°C. The cells were incubated with goat anti-mouse IgG conjugated with Alexa Fluor®-488 or 568 (1:333 dilution, Molecular Probes), for 1 hr at RT followed by nucleic acid staining with 0.1% DAPI. Cultured HEK-293 cells were used as a negative control (Data not shown). The images were taken by Q-image camera coupled with BX60 Olympus microscope.

To identify the uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (Dil-Ac-LDL) by MEC, the fixed and permeabilized cells were incubated with Dil-Ac-LDL for (1:100; Invitrogen, Carlsbad, CA) 4 hr followed by acquisition of imaging.

Capillary-like tube formation. The cells isolated using the two-step scheme were seeded in 6-well plates coated with Matrigel™ (BD Biosciences, Franklin Lake, NJ) in accordance with the manufacturer's instructions for EC functional characterization. Tube formation was observed and images were acquired by phase contrast microscopy (IX71 Olympus Microscope) 4 or 8 h after the cells were seeded in the plate.

2.4. Flow Cytometry

The cell pellet was suspended in 2% paraformaldehyde in Dulbecco's phosphate buffered saline (DPBS) for 10 min at 37°C for fixation. The cells were blocked using 5% (w/v) BSA for 1 hr at RT followed by incubation with mouse monoclonal antibody against CD31/PECAM-1 (ABD Serotec, catalogue MCA1334G, 1:30 dilution) in 0.5% BSA/DPBS overnight at 4°C with constant rotation. After washing, the cells were labeled with secondary antibody, which was goat anti-mouse IgG conjugated with Alexa Fluor®-488 (1:333 dilution), for 30 min with constant rotation. The negative control was prepared in the same way as described above except the omission of primary antibody labeling. Subsequently, all the samples were assessed by flow cytometry (Accuri™ 6C, BD Bioscience, Franklin Lake, NJ). The cells labeled with primary-secondary antibody-Alexa Fluro®-488 were detected and the data were analyzed by its software.

2.5. Statistics

Purification of MEC derived from C57BL/6J and $P2Y_2R^{-/-}$ mice was analyzed by using student's t-test (GraphPad Prism version 5, GraphPad Software, San Diego, CA). The level of significance was set at 0.05

(p < 0.05).

3. RESULTS

3.1. Morphology of Primary Endothelial Cells

The morphological properties of cultured primary mouse MEC (Figure 1A) were compared with both primary cultured rat MEC (Figure 1B) as well as human umbilical vein endothelial cells (HUVEC, purchased from Lonza, Figure 1C).

3.2. Identification of Expression of CD31/PECAM-1 of Endothelial Cell Marker

CD31/PECAM-1 has been widely used as an endothelial cell marker. The immunofluorescence assay with monoclonal antibody specifically against CD31/PECAM-1 showed positive staining of CD31/PECAM-1 in cells isolated by the two-step method ($n = 3$). The representative images of the expression of CD31/PECAM-1 in isolated cells are shown in Figures 2A-C. The positive control that was used for immunofluorescence in the isolated cells, was demonstrated by in vivo staining endothelial cells of an intact venule in cramaster skeletal muscle, shown in Figure 2D. Additionally, as a comparison, primary rat MEC stained by CD31/PECAM-1 were shown in Figure 2E.

3.3. Identification of Physiological Function of Microvascular Endothelial Cells

Uptake of acetylated low density of lipoprotein (*Ac-LDL*). Uptake of Ac-LDL is one of the physiological properties of EC. This property of EC was demonstrated in murine MEC in Figures 3A-C using DiI-labeled Ac-LDL. Again, uptake of DiI-Ac-LDL by rat MEC and HUVEC is displayed in Figure 3D and Figure 3E, respectively, as positive controls.

Capillary-like tube formation. A capillary-like tube formation assay was performed to assess the property of angiogenesis for MEC. The cells isolated and purified by using GS-I and antibody against CD31/PECAM-1 were able to form capillary-like tubes, shown in Figure 3F.

3.4. Detection and Count of Endothelial Cells by Flow Cytomery

The purified cells were labeled with CD31/PECAM-1 antibody and analyzed by flow cytometry. The cells labeled with only secondary antibody conjugated with fluorescence, but omission of primary antibody served as a negative control. The representative data from the flow cytometry in three cell samples derived from one culture dish (defined $n = 1$) are shown in Figures 4A-E. The count of CD31/PECAM-1 positive cells in primary cultured cell population was 88.02% ± 2.99% ($n = 6$) and 87.78% ± 6.78% ($n = 6$) for cells derived from wild type C57BL/6J mice and P2Y$_2$ knockout mice, respectively, shown in Figure 4F.
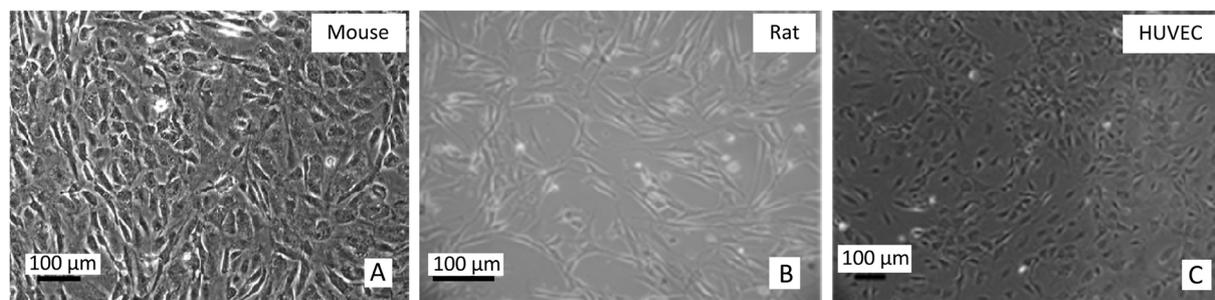

Figure 1. Representative morphology of primary and cultured mouse MEC derived from abdominal skeletal muscles (A). MEC derived from different species (rat), but the same tissue (abdominal skeletal muscle) (B) and human umbilical vein EC (HUVEC) (C) commercially purchased are presented for reference.
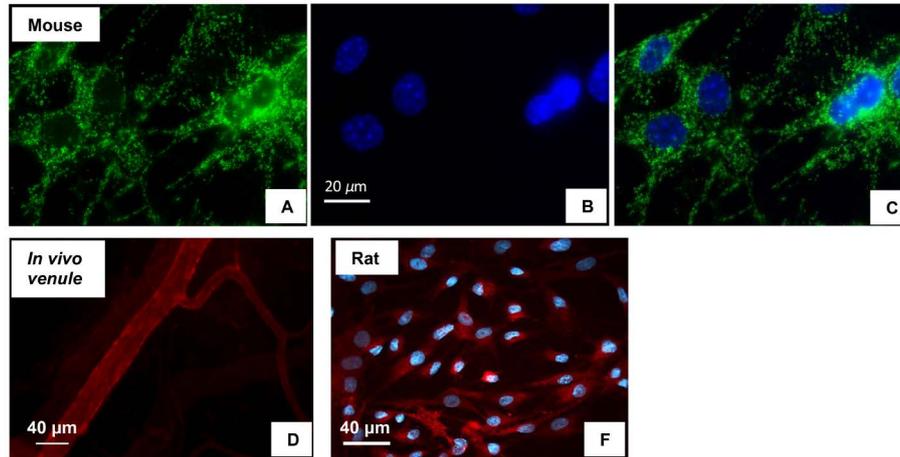
Figure 2. Representative images of immunofluorescence for identification of CD31/PECAM-1 expression in murine primary cultured EC (A-C) and *in vivo* murine venules (D). The green (A, B, and C; labeled with Alexa-488) and red color (D and E, labeled with Alexa-568) indicate expression of CD31/PECAM-1. The blue color (B, C, and F) denotes the cell nuclei stained with DAPI. The scale of image A-C is shown in image B. Rat primary cultured EC derived from the same tissue (abdominal skeletal muscle) served as a positive control (E).
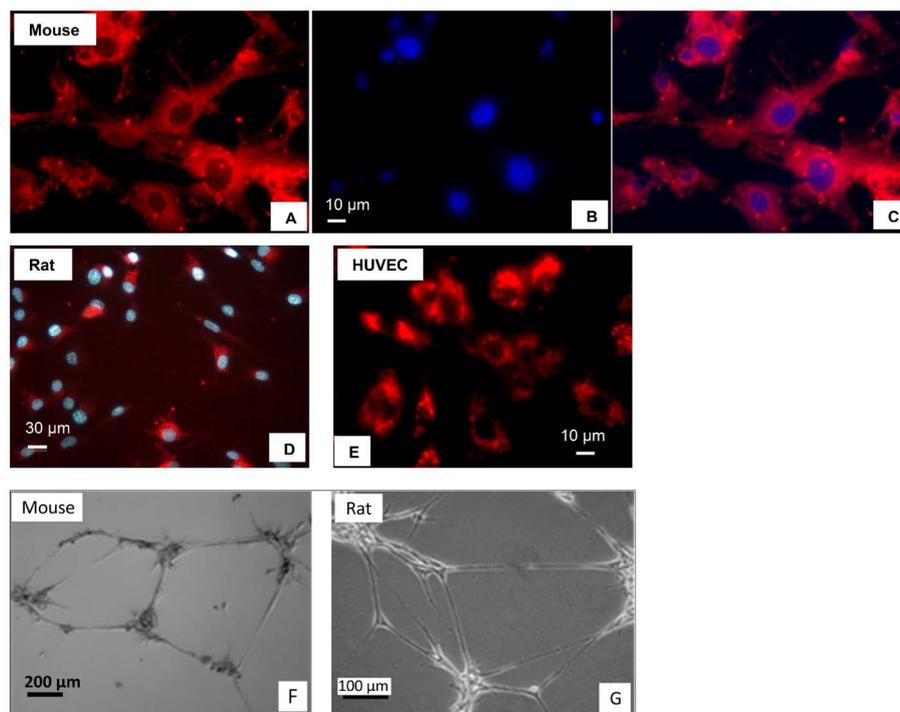

Figure 3. Physiological function assay of MEC. The representative images of uptake of DiI-Ac-LDL by primary cultured murine MEC is demonstrated in images of A-C. The uptake of DiI-Ac-LDL by primary cultured rat EC (D) and HUVEC (E) served as positive controls. The blue color denotes nuclei stained with DAPI. The scale for the images from A to C is shown in B. The representative images of capillary-like tube formation, a unique intrinsic property of MEC, are shown in F (murine MEC) and G (rat MEC).
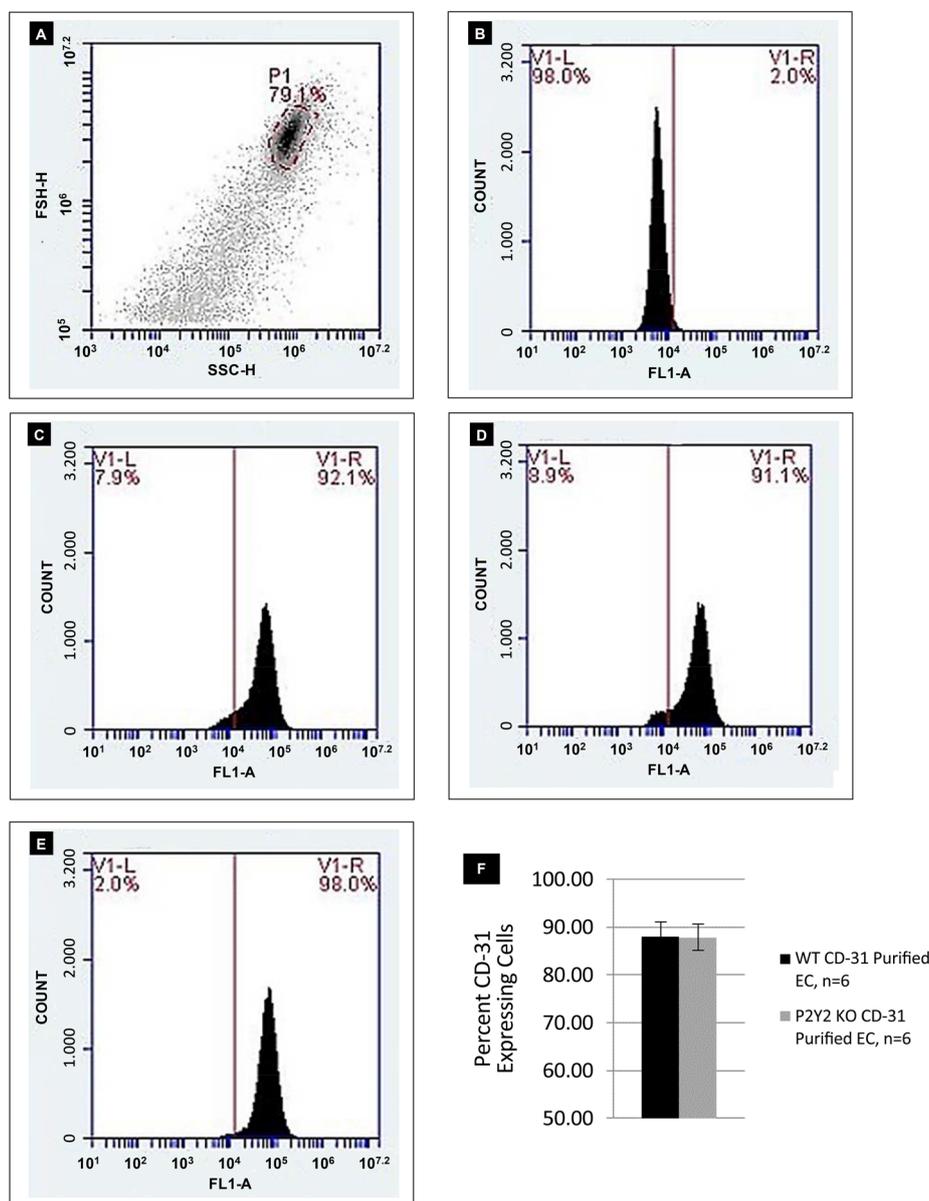
Figure 4. Representative data of flow cytometry for detection and enumeration of CD31/PECAM-1 positive cells (A-E). The gate (A) of the cell population was applied for all of the samples. The cells incubated with secondary antibody alone served as a negative control (B) for the three separate samples labeled with both primary and secondary antibodies (C-E). The count of CD31/PECAM-1 positive cells isolated and purified by the two-step scheme using Dynabeads™-GS-I lectin followed by Dynabeads™-CD31 monoclonal antibody is shown in F. The purity of isolated cell populations from skeletal muscle microvessels was 88.02% ± 2.99% ($n = 6$). WT indicates cells derived from wild type C57BL/6J mice and P2Y$_2$ KO presents the cells from P2Y$_2$ receptor knockout mice.

4. DISCUSSION

We report a refined method to isolate MEC derived from murine skeletal muscle. This method employed the use of a Dynabeads™-based two-step scheme using GS-I, to recognize the carbohydrate moiety

and then monoclonal antibody against CD31/PECAM-1 following enzymatic dissociation of skeletal muscles. To the best of our knowledge, this is the first description to use combined methods to isolate MEC from murine skeletal muscles. Such a refined method met the requirements of simple and fast preparation, high yield and purity of MEC population, and consistency.

4.1. Enzymatic Dissociation of Skeletal Muscle Tissue

The enzyme-based tissue digestion possibly affects the structure or negatively impacts function of the surface molecules expressed on the cells. The enzymatic digestion of tissue has been extensively used for the isolation of cells from intact tissue. It was found that dispase at 0.8 U/ml (equivalent to 1 mg/ml) for 45 seconds at 37°C with 5% fetal calf serum affected the expression of numerous surface molecules and impaired antigen-mediated detectability of the majority of surface markers in immune cells [13]. To overcome this problem, we first detected abundant surface carbohydrates which attach to the surface proteins and lipids on the membrane of MEC [14]. The expression level of carbohydrates is much greater than that of a surface antigen, such as CD31/PECAM-1, because CD31/PECAM-1 is only one of various surface proteins in MEC [15].

4.2. Isolation of MEC Using Dynabead-Based Two-Step Scheme

We reported the modified method with the two-step isolation scheme here. The first step was to use GS-I to bind a carbohydrate moiety specifically expressed on the surface of microvascular endothelial cells [16, 17]. The carbohydrate chains are covalently attached to the surface proteins or lipids, and these are abundantly present on the EC surface as components of the glycocalyx [14]. The glycocalyx is a thick coating on the surface of endothelial cells, demonstrated *in vivo* from microvascular endothelial surface in skeletal muscles [18]. It was demonstrated that GS-I specifically bound to microvascular endothelial cells, but not macrovascular endothelial cells [16]. Using GS-I to isolate MEC has been used in various physiology or pathophysiology studies focusing on MEC [10, 12, 19, 20]. The first step using GS-I to isolate MEC enabled us to obtain abundant cells, providing sufficient numbers of isolated cells. Unfortunately, GS-I was also found to bind to non-endothelial cells such as epithelia cells [16].

To further remove non-endothelial cells from the cell population we employed Dynabeads coated with monoclonal antibody specifically against CD31/PECAM-1. CD31/PECAM-1 has been recognized as an endothelial marker and is extensively used for the detection or isolation of vascular EC. As described above, cell surface markers such as CD31/PECAM-1 can be degraded by enzymes in the process of enzymatic dissociation of tissue [13]. Thus, to avoid this issue, we targeted CD31/PECAM antigen following subculture of the cells after initial isolation via the GS-I agglutination method. The specificity of CD31 antibody used in this study was validated by in vivo immunofluorescence staining in a venule of murine cremaster muscle, shown in Figure 2D. It was found that CD31/PECAM-1 is also expressed at low level on platelets and leukocytes, in addition to EC [21]. Thus, this study combined two strategies, by using GS-I followed by CD31-based antibody isolation to improve the purity of primary cultured MEC.

4.3. Characterization of Primary Endothelial Cells

The identification of EC, including cobblestone morphology, immunostaining of EC biomarkers, and physiological functions specific for MEC such as uptake of DiI-Ac-LDL and promotion of angiogenesis, has been performed in this study. Although the high level of DiI-Ac-LDL uptake has been extensively used for identification of EC, other cells, such as macrophages, can also take up DiI-Ac-LDL [22]. Thus, several lines of evidence to characterize the isolated cells in the study overcomes the problems that other cells share markers or functional properties with EC. Flow cytometry revealed the percentage of CD31 positive cells in the population of all cells to be about 90% as shown in Figure 4. The reduced florescence intensity of CD31 (90%, but not 100%) was, in part, likely caused by steric hindrance on the part of the antibody [23].
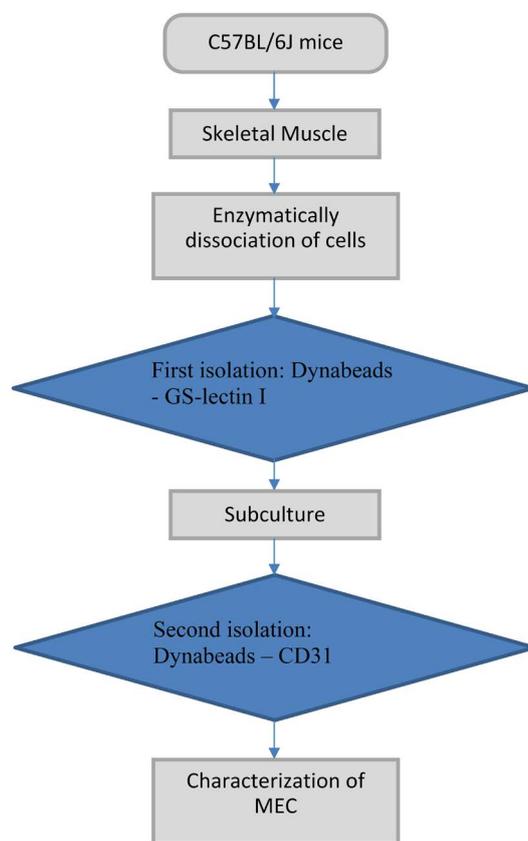
Figure 5. A schematic diagram of the two-step scheme method for efficient isolation of MEC from murine skeletal muscles.

4.4. Limitation of the Study

The study used GS-I and CD 31 to isolate cells so that MEC are possibly the mixed endothelial cells derived from arterioles, capillaries, and venules.

5. CONCLUSION

A summary of the isolation and characterization of primary microvascular endothelial cells illustrated in the manuscript is shown in Figure 5. This study demonstrates a simple, effective method to acquire primary cultured MEC derived from skeletal muscles in any murine model, such as those genetically engineered or various disease models. Characterization of those cells will help elucidate the mechanisms of diseases associated with dysfunctional microvascular endothelial cells, resulting in novel strategies for the treatment.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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