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Isolation and Identification of Vascular Endothelial Cells from Distinct Adipose Depots for Downstream Applications

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Abstract

Vascular endothelial cells lining the wall of the vascular system play important roles in a variety of physiological processes, including vascular tone regulation, barrier functions, and angiogenesis. Endothelial cell dysfunction is a hallmark predictor and major driver for the progression of severe cardiovascular diseases, yet the underlying mechanisms remain poorly understood. The ability to isolate and perform analyses on endothelial cells from various vascular beds in their native form will give insight into the processes of cardiovascular disease. This protocol presents the procedure for the dissection of mouse subcutaneous and mesenteric adipose tissues, followed by isolation of their respective arterial vasculature. The isolated arteries are then digested using a specific cocktail of digestive enzymes focused on liberating functionally viable endothelial cells. The digested tissue is assessed by flow cytometry analysis using CD31+/CD45− cells as markers for positive endothelial cell identification. Cells can be sorted for immediate downstream functional assays or used to generate primary cell lines. The technique of isolating and digesting arteries from different vascular beds will provide options for researchers to evaluate freshly isolated vascular cells from arteries of interest and allow them to perform a wide range of functional tests on specific cell types.

Introduction

Endothelial cells are well recognized for their important roles in a variety of physiological processes, including barrier functions, angiogenesis, and vascular tone regulation[1,2]. Although endothelial cell dysfunction is well documented in promoting atherosclerosis, hypertension, diabetes, etc., the underlying mechanisms driving endothelial dysfunction remain poorly understood and likely differ between distinct vascular beds[2,3,4]. The effort to unravel these pathological

mechanisms of endothelial cell dysfunction is challenged by the limited access to a pure population of endothelial cells from tissues and/or the phenotypic changes of endothelial cells in culture[5,6]. Therefore, being able to isolate and perform analyses on endothelial cells from various vascular beds in their native form will give insight into the processes of cardiovascular disease.

This protocol presents the procedure to dissect subcutaneous and mesenteric adipose tissues from mice, followed by isolation of their respective arterial vasculature. Functionally viable endothelial cells lining the arterial walls are liberated using a specific cocktail of enzymes. Special care was taken to optimize the conditions of the digestion protocol to obtain a sufficient yield of endothelial cells from <1 mg of starting tissue while keeping biomolecular markers intact for analysis. Isolated endothelial cells are next identified using flow cytometry. The presence of CD31 (PECAM) is used to primarily identify endothelial cells. Due to the expression of CD31 in other cell types, including several of hematopoietic origin that also express CD45, the purity of the isolated endothelial cells was further enhanced by the exclusion of cells expressing both CD31 and CD45[5,6,7,8]. Moreover, depending on the research question and the downstream applications to be employed, researchers should consider selecting an extensive panel of positive and negative selection markers to optimize the purity of the cell population of interest.

Though the technique of dissecting and isolating adipose depot arteries has been used in the previous publications[9,10,11,12,13], a detailed protocol describing the isolation of embedded arteries has yet to be presented. Demonstrating the technique for the isolation and digestion of arteries from different vascular beds from a given species of interest will provide options for researchers to evaluate freshly isolated vascular cells from arteries of interest and allow them to perform a wide range of functional tests on specific cell types. Tests may include but are not limited to flow cytometry for cell sorting and membrane protein expression[5,8], electrophysiology for ion channel activity[9], molecular profiling (proteomics/genomic analyses, etc.)[14,15], and the generation of primary cell lines for drug screening *in vitro*[16,17].

Protocol

The use of animals in these studies was approved by the University of Delaware, Institutional Animal Care and Use Committee (#1372).

1. Tissue dissection and cleaning

NOTE: 10- to 12-week-old C57BL/6J mice are used in the video protocol. Please refer to **Figure 1** for the schematic and the desired outcome of tissue dissection and cleaning arteries and refer to the **Table of Materials** for the list of supplies and manufacturer information.

1. Euthanize the mouse by CO_2 asphyxiation followed by cervical dislocation.
2. Secure the mouse using dissection pins and spray the ventral surface with 70% ethanol.
3. Isolation of subcutaneous adipose tissues located by each hindlimb

   NOTE: Dissecting tools needed: straight Bonn scissors (9 cm) and blunt, curved Graefe forceps.

   1. Use forceps to lift the skin right above the genitals and make a small (2 mm) incision in the skin.

2. Insert the tip of the scissors into the incision site and make a 4-5 cm midline incision, beginning at the initial incision and dissecting cranially to the base of the sternum. Be cautious to not penetrate the abdominal cavity.
3. Make a 1 cm lateral incision, extending laterally from the midline immediately below the forelimb.
4. Make a diagonal 1 cm incision between the hindlimb and genitals.
5. Make small cuts along the midline skin incisions to peel away the associated connective tissue and expose the subcutaneous adipose depot. Pin the skin down.
6. Identify the "C-shaped" subcutaneous adipose tissue and artery/vein that runs perpendicular to the abdominal cavity.
7. Start from the bottom of the C shape near the genitals and gently grasp the adipose tissue to expose the connective tissues. Make small cuts in the connective tissue to separate the fat from the skin. Avoid disturbing the exposed vasculature.
8. Continue dissecting away the connective tissue until the subcutaneous adipose tissue can be removed intact. Carefully separate the exposed artery from the surrounding connective tissue.
9. Store the isolated subcutaneous adipose in HEPES buffer on ice until ready to isolate the vascular bed of interest.
10. Repeat steps 1.3.3.-1.3.5. for the remaining posterior subcutaneous adipose depot.

4. Isolation of the mesenteric adipose depot

NOTE: Dissecting tools needed: straight Bonn scissors (9 cm), Graefe forceps, a pair of #5 forceps, straight iris scissors, and curved Bonn scissors (9 cm).

1. Use the Graefe forceps to lift the thin peritoneal cavity wall above the urinary bladder and make a small incision using straight scissors.
2. Slowly lift the penetrated peritoneal cavity wall, carefully insert the straight iris scissors into the incision site, and make a 4-5 cm incision from the urinary bladder to the sternum.
3. Make two 1 cm long horizontal incisions with the straight iris scissors, extending laterally from the midline to immediately above the hindlimb and below the forelimb. Use the Graefe forceps to peel back the abdominal musculature and expose the peritoneal viscera.
4. Repeat step 1.4.3. on the opposite side.
5. Use the pair of #5 forceps to lift the intestines out of the visceral cavity to reveal the mesenteric adipose.
6. Use the curved scissors and #5 forceps to separate the adipose along the colon, starting from the caecum to where the colon descends from view.
7. Return to the caecum and use the curved scissors and #5 forceps to separate the adipose along the small intestine to the pancreas. Remove a small part of the pancreas to completely isolate the mesenteric adipose.

   NOTE: Removing a small part of the pancreas along with the mesenteric adipose may help in cleaning the arteries as it provides stability during cleaning.

8. Store the isolated mesenteric adipose in HEPES buffer on ice until ready to isolate the mesenteric arteries.

5. Isolation of the respective arteries from subcutaneous and mesenteric adipose tissues

NOTE: Dissecting tools needed: a dissecting dish and stereoscope with a light source, a pair of #55 forceps, and a pair of #5 forceps.

1. Place ~10 mL of cold HEPES buffer into the dissecting dish and transfer the adipose tissue to the dish using the #5 forceps.

2. Use the stereoscope at 1x magnification to view and position the adipose tissue to expose the artery/vein pair(s) (**Figure 2**).

3. Use the #55 forceps to carefully remove the parenchymal adipose from the artery. Remove small pieces of adipose at a time and carefully observe artery/vein pairs under the stereoscope to identify and avoid damaging the vasculature of interest.

NOTE: Adjust the magnification and light source accordingly to clean the arteries. Increase the magnification and light intensity to better view the arteries as adipose is removed. Fine cleaning of the arteries should be done under 4x-5x magnification. It is important to distinguish between connective tissues (collagens), veins, and arteries. Connective tissues and collagen fibers have a string-like appearance without branches, are striated, and have a whitish color. Unlike collagen fibers and connective tissues, arteries and veins are transparent and have multiple branches with a defined lumen. Small arteries and veins may appear similar in appearance when free of parenchymal tissue. The presence of blood in the lumen can further help one to identify the arteries from veins. Veins have thinner blood vessel walls and a larger lumen and contain more blood compared to comparably sized arteries.

4. Repeat step 1.5.3. until the arteries are completely void of parenchymal adipose tissue.

5. Use #5 forceps to transfer the cleaned arteries to a new tube with fresh HEPES buffer and keep on ice until ready for digestion.

2. Digestion of isolated arteries for isolation of endothelial cells

NOTE: Please refer to **Figure 2** for the schematic of tissue digestion and isolation of endothelial cells and the **Table of Materials** for a complete list of supplies needed for the protocol.

1. Add 1 mg each of dispase and elastase to each 2 mL microcentrifuge tube. Add 2 mL of dissociation solution to each tube. Vortex and incubate at 37 °C until completely dissolved. Use #5 forceps to transfer the cleaned arteries (step 1.5.5.) to the respective sample tubes. The final concentration of each enzyme is 0.5 mg/mL.

2. Incubate at 37 °C for 1 h with gentle agitation by inversion every 10-15 min, such that the arteries are suspended in solution to maintain consistent and uniform contact between the enzymes and arteries.

3. Weigh 1 mg of collagenase type I per sample and add it to new tubes.

4. Allow the arteries to settle at the bottom of the tube. Remove 500 µL of the buffer from the top without disturbing the arteries and transfer it into designated tubes containing collagenase. Vortex and return the

solution to the respective samples in the original sample tubes. The final concentration of collagenase type I is 0.5 mg/mL.

5. Incubate at 37 °C for 15 min. Ensure that the arteries separate into pieces following brief shaking of the sample tube. If the arteries do not separate into pieces following perturbation, incubate in 5 min increments until the arterial breakdown is visible.

6. Using a glass pipette, vigorously triturate the digested tissue 10x-15x to mechanically dissociate the cells.

7. Pass the solution and digested tissue through a 70 µm cell strainer or a flow cytometry tube strainer to obtain single-cell suspensions.

3. Staining of endothelial cells prior to flow cytometry

NOTE: The staining and processing of endothelial cells are carried out on ice to improve cell viability, unless instructed. The 5 mL polystyrene round-bottom tubes are centrifuged at 1,163 x *g* for 3 min at room temperature (RT).

1. Centrifuge the single-cell suspensions at 1,163 x g for 3 min at RT.

2. Remove the supernatant and resuspend the cell pellet in 1 mL of PBS + 5% BSA for 30 min at RT.
   NOTE: Researchers should consider blocking FC receptors depending on the cell type, target of interest, and antibody used[18,19].

3. Add 1 µL of cell viability stain (405 nm excitation) (**Table of Materials**) and incubate in the dark for 30 min at RT.

4. Centrifuge the cell suspension at 1,163 x *g* for 3 min at RT. Resuspend the cell pellet in 200 µL of PBS + 1% BSA.

5. To the samples, add primary antibodies conjugated to CD31 (CD31-PE, 0.75 µg/sample) and CD45 (CD45-FITC, 2.5 µg/sample) and incubate on a rocker in the fridge for 15 min after covering the samples with aluminum foil.
   NOTE : To identify and/or obtain a pure population of endothelial cells, probe for both CD31 and CD45 using CD31- and CD45-conjugated primary antibodies with distinct excitation/emission fluorescence and gate/sort cells with a $CD31^{+}CD45^{-}$ expression profile. Compensation may be required depending on the fluorophores used[18,20,21].

6. Wash the cells by adding 1 mL of PBS + 1% BSA directly to the cell suspension.

7. Centrifuge the cell suspension at 1,163 x *g* for 3 min at RT. Remove the supernatant and resuspend in 200 µL of 1% formaldehyde with 0.1% BSA. Store in the fridge covered with aluminum foil until ready for flow cytometry.
   NOTE: Cell yields are improved by reducing the wash and centrifugation steps. These may need to be altered depending on the outcome approach. The sample in fixative should be analyzed within 24 h.

Representative Results

A schematic of the workflow is shown in **Figure 1**. The schematic highlights the protocol steps described in more detail in the protocol text. **Figure 2** shows pictures of subcutaneous adipose (**Figure 2A, left**) following dissection and a subcutaneous artery arcade (**Figure 2A, right**) following cleaning of the parenchymal tissue. **Figure 2** also shows the mesenteric adipose (**Figure 2B, left**) following dissection and the mesenteric arterial arcade (**Figure 2B, right**) following cleaning of the parenchymal tissue.

The protocol presented here is designed to assist researchers potentially interested in a) comparing distinct adipose depot vasculature beds in basic science approaches and/or disease models, b) the digestion of vascular beds prior to isolation of vascular cells of interest for downstream application, and c) the use of flow cytometry to identify vascular cells of interest (**Figure 3**) for a variety of applications including, but not limited to, protein expression analyses as performed here (**Figure 4**). **Figure 3** details an example of our approach to identify endothelial cells from digested mouse arteries using flow cytometry. Cell preparations obtained from digested subcutaneous (**Figure 3A**) or mesenteric (**Figure 3B**) adipose arteries were stained with CD31-PE, CD45-FITC, and a cell viability dye prior to the fixation and identification of viable CD31+CD45− endothelial cells using flow cytometry, as similarly performed elsewhere[8]. The cell viability stain allowed for the identification of only those viable cells that survived the isolation and digestion protocol prior to fixation. The use of this method will allow researchers to assess the expression or function of viable vascular cells of interest.

To determine if endothelial cells isolated from distinct adipose depot vasculature exhibited differences in membrane protein expression, isolated cells were probed for the fatty acid translocase, CD36 (**Figure 4**), as this membrane protein was recently shown to be essential in the endothelial-mediated distribution of fatty acids to tissues[22]. Therefore, determining the relative expression differences between membrane CD36, for example, in distinct vascular beds may a) support existing data regarding differential preference for fatty acid utilization among different tissues and/or b) unveil potential novel differences in tissue distribution of fatty acids in health and disease. From the same preparations of digested vascular cells exemplified in **Figure 3**, CD31+CD45−CD36+ endothelial cells were identified in subcutaneous (**Figure 4A**) and mesenteric (**Figure 4B**) adipose, and CD36 expression was quantified in this population in each vascular bed. **Figure 4C** and **Figure 4D** reveal that both the percentage of endothelial cells expressing CD36 and the intensity of CD36 expression were greater in subcutaneous endothelial cells compared to that observed in mesenteric endothelial cells. These preliminary findings support the use of our methodology to identify distinct differences between vascular beds.

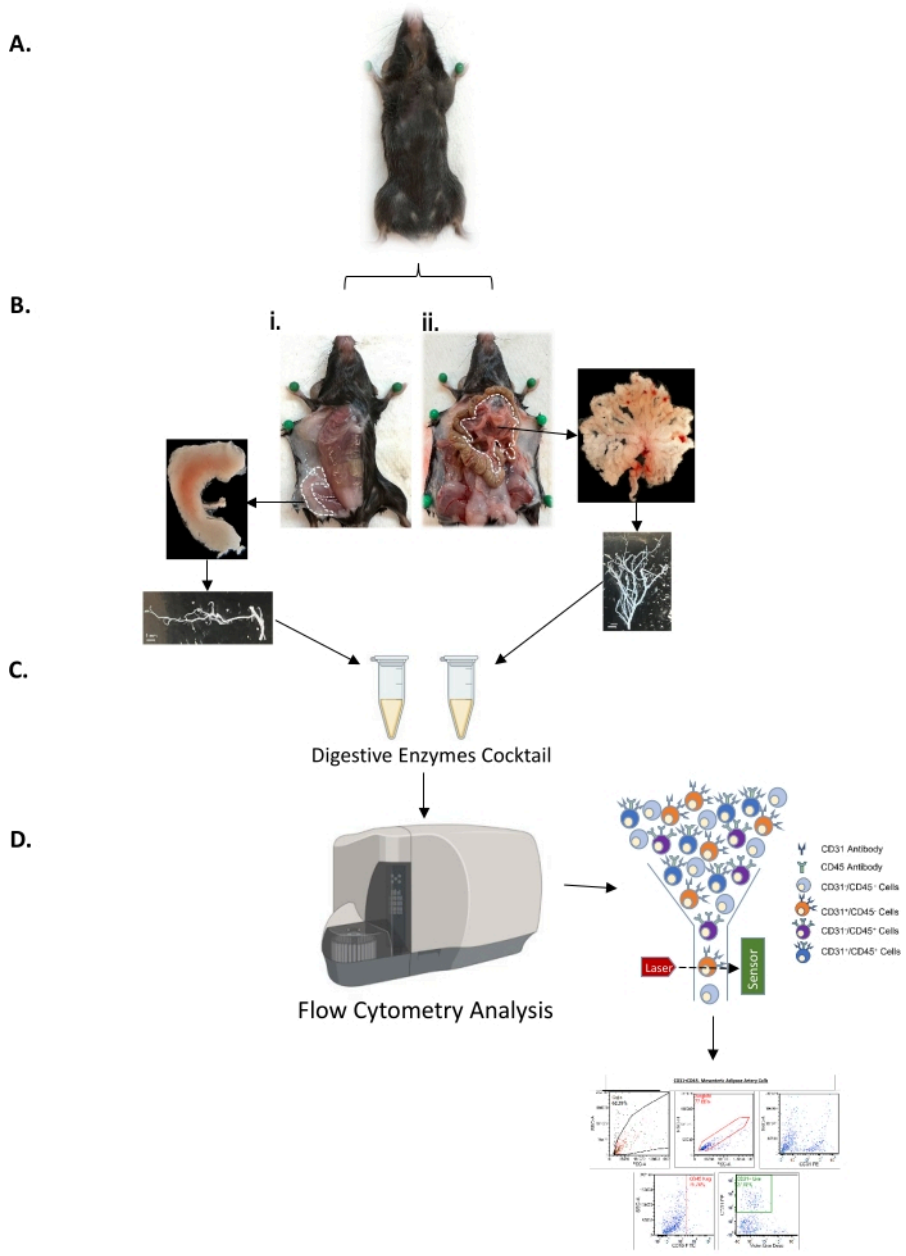

Figure 1: Working scheme to isolate endothelial cells from subcutaneous and visceral adipose tissues for downstream applications. (**A**)**10**-12-week-old C57BL/6J mice were euthanized and used to demonstrate this procedure. (**Bi**) First, the subcutaneous adipose is removed. (**Bii**) Then, the mesenteric (visceral) adipose tissue is subsequently removed. The white dashed lines indicate the locations of the subcutaneous (left) and mesenteric (right) adipose depots following the dissection and removal. The respective arteries are isolated for downstream applications. (**C**) In this protocol, isolated arteries are next digested using a specific enzyme cocktail as a first step in liberating functionally viable endothelial cells. (**D**) Isolated endothelial cells are identified by probing for CD31+ and CD45− cells using conjugated antibodies prior to

flow cytometry analysis. Cells with an expression profile of $CD31^{+}/CD45^{-}$ are the endothelial cells of interest for additional analyses. Please click here to view a larger version of this figure.

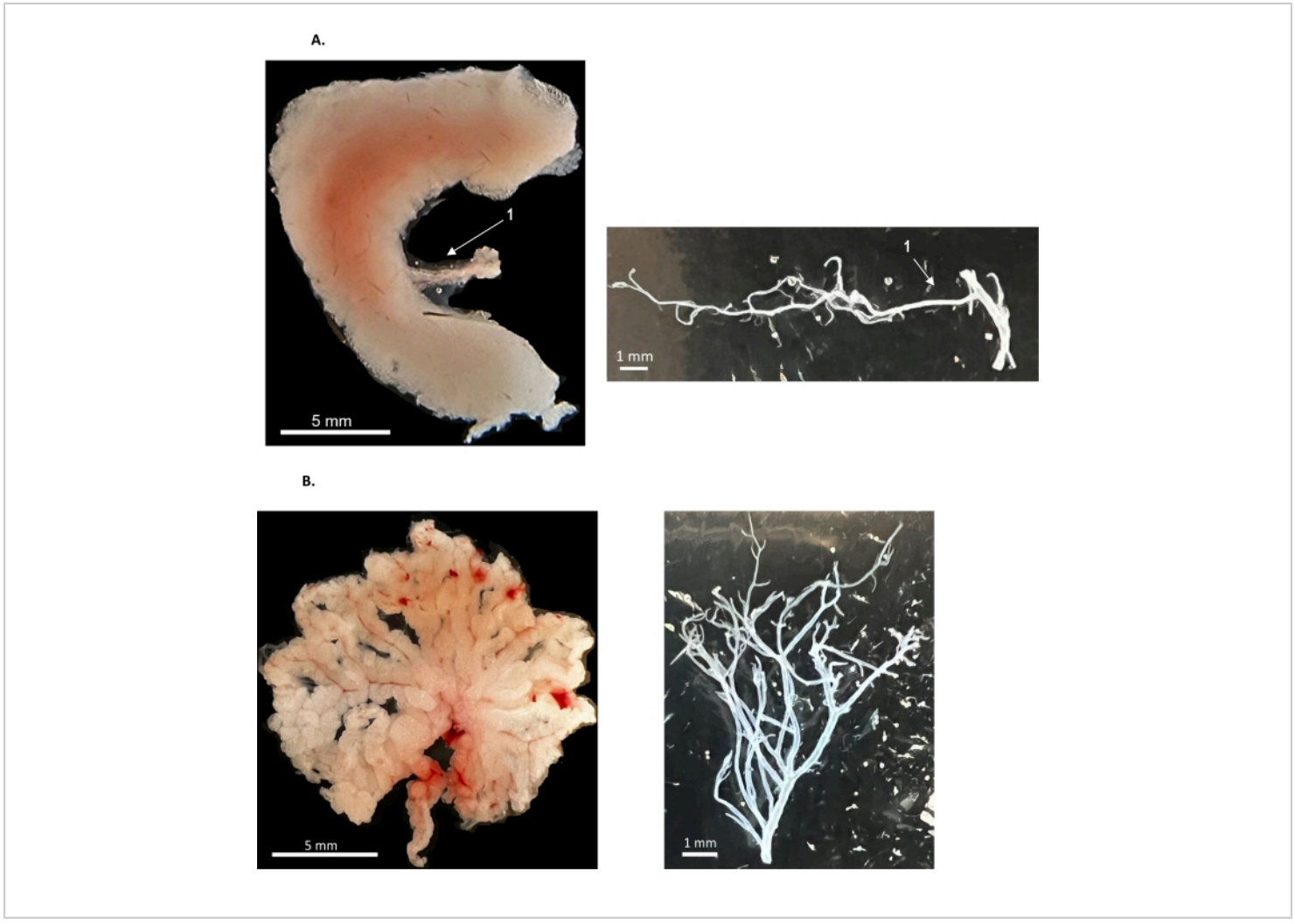

Figure 2: Isolated subcutaneous and visceral adipose tissues and respective arteries. (**A**) Left: Isolated "C-shaped" subcutaneous adipose tissue from hindlimbs. A major branch of the subcutaneous adipose artery, denoted by arrow (1), is revealed when parenchymal adipose is removed. Right: Isolated subcutaneous adipose arteries. (**B**) Left: The mesenteric (visceral) adipose tissue is isolated from the gut. Right: The respective arterial arcade is isolated by removing the parenchymal tissue. Different scales are used between the pictures of adipose (5 mm) and arteries (1 mm). Please click here to view a larger version of this figure.

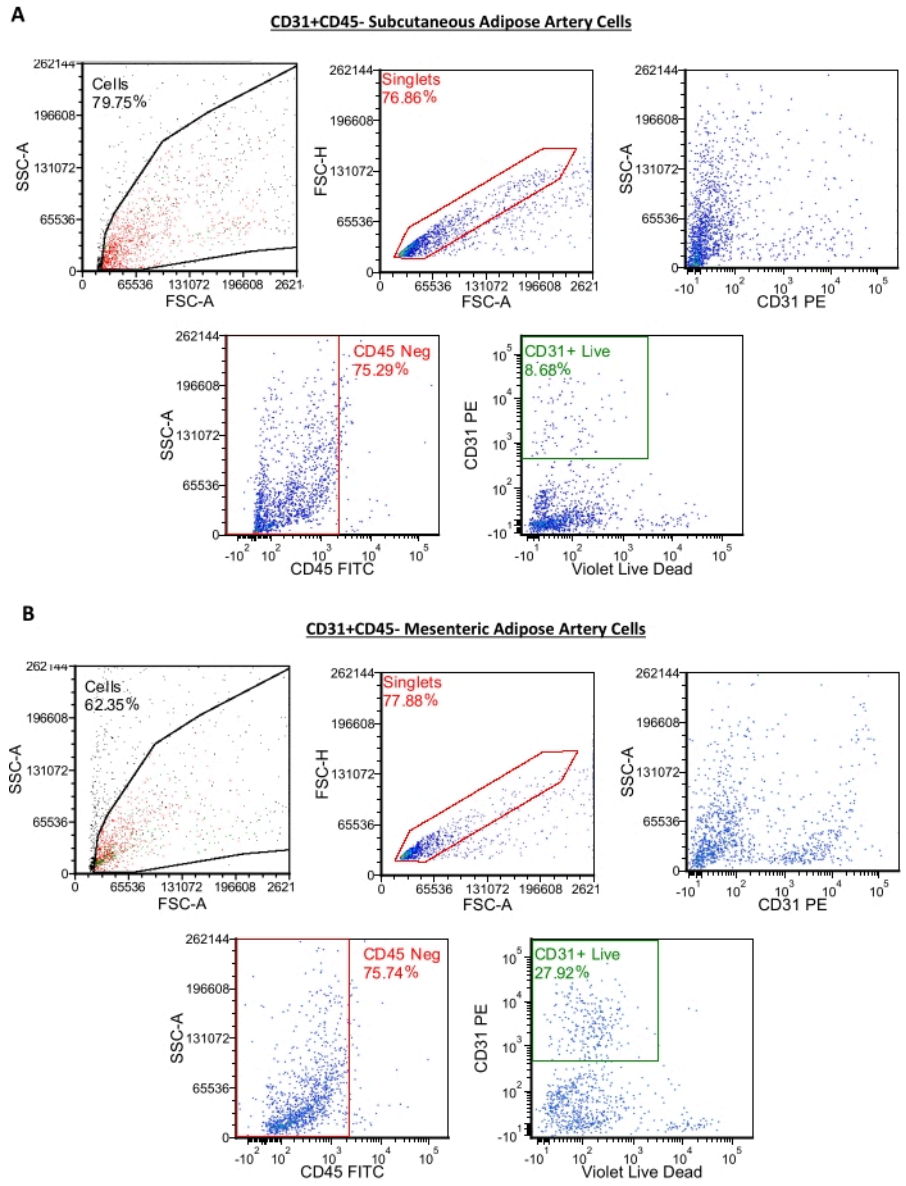

Figure 3: Identification of endothelial cells following arterial tissue digestion via flow cytometry. Representative plots showing cells liberated from isolated (**A**) subcutaneous or (**B**) mesenteric adipose arteries. Cells were exposed to conjugated antibodies that targeted extracellular epitopes of CD31 (PE) and CD45 (FITC) prior to fixation. Flow cytometry was used to identify the CD31+CD45− endothelial cell population. A cell viability stain was used to select only the cells that survived the isolation and digestion protocols for subsequent analyses. In addition, appropriate gating at this stage will further separate an intended cell population from contaminating cells should the size of the cell type of interest be known. Please click here to view a larger version of this figure.

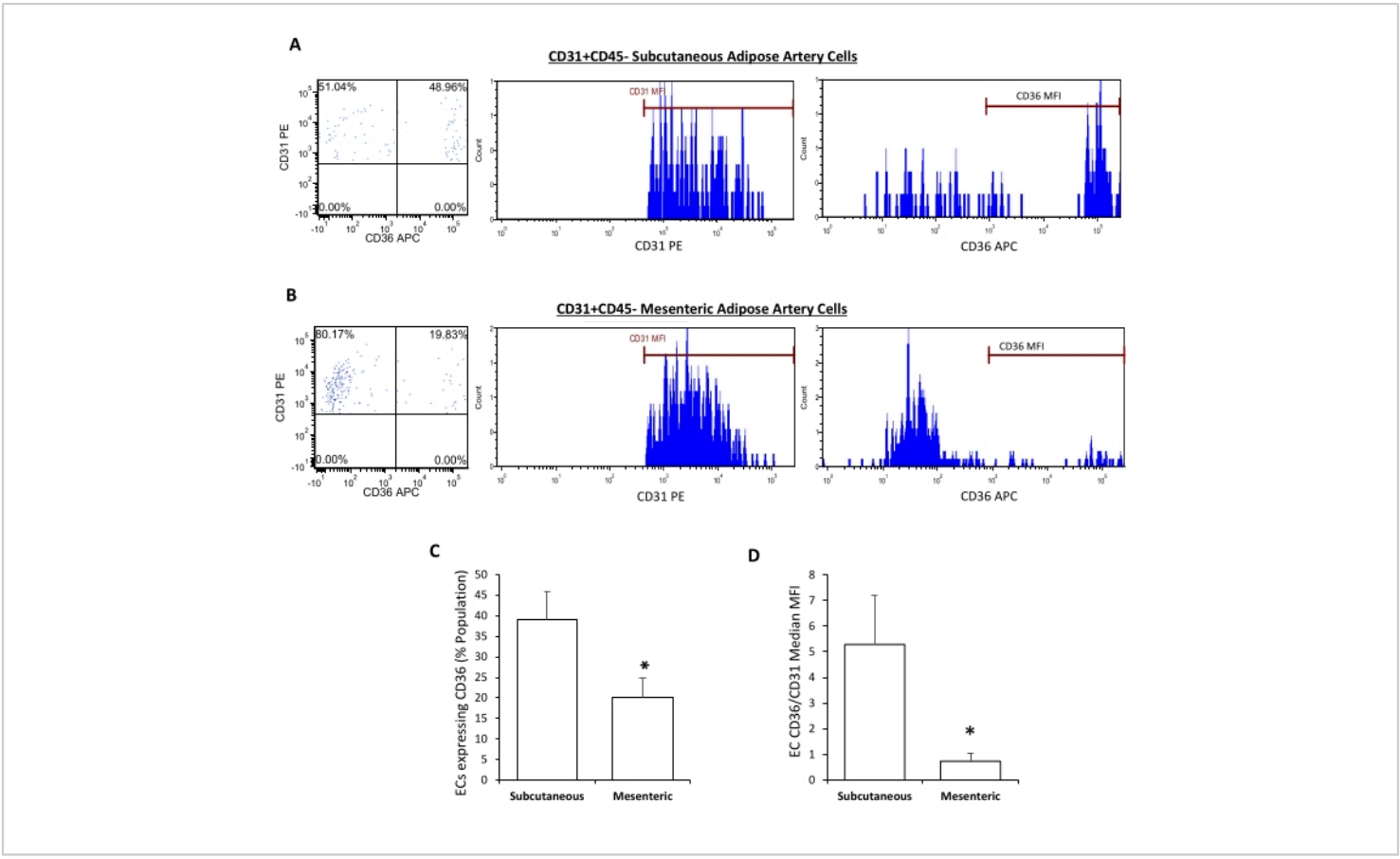

Figure 4: Analysis of CD36 membrane expression in subcutaneous vs. mesenteric adipose artery endothelial cells via flow cytometry. Representative population plots and histograms showing endothelial CD36 (APC) membrane expression in (**A**) subcutaneous or (**B**) mesenteric adipose arteries. (**C**) Percentage of endothelial cells (ECs) expressing CD36 in subcutaneous vs. mesenteric ECs (n = 5, 3 males, 2 females; *p < 0.05 following Student's t-test). (**D**) Normalized EC membrane CD36 expression in subcutaneous vs. mesenteric adipose arteries (n = 5, 3 males, 2 females; *p < 0.05 following Student's t-test). Please click here to view a larger version of this figure.

Discussion

Endothelial dysfunction is a precursor to severe disease states, likely driving the development of atherosclerosis, hypertension, and stroke[3,23]. While the identified mechanisms underlying endothelial dysfunction in a given pathological condition are many, distinct vascular beds are likely to be differentially influenced by pathological conditions[4,24]. Furthermore, different cardiovascular risk factors (e.g., obesity, hypertension, dyslipidemia, smoking, diabetes) induce dysfunction through a variety of distinct mechanisms[23,25]. Therefore, it is critical to isolate endothelial cell populations from established animal models of disease or accessible human tissue and perform assays on cells immediately removed from the *in vivo* environment. Isolating cells in this way has a unique advantage over studying cells in culture in that they are void of culture-induced phenotypic changes[5,6]. Moreover, including a heterogenous endothelial cell population, as observed *in vivo*[26,27] (and

that can be further separated using flow-assisted cell sorting), from a live organism better informs the *in vivo* environment. Finally, this method is applicable to the investigation of numerous animal models and potentially human tissue and can be used to generate primary cell culture lines, if such a need is warranted.

The critical step in this protocol, and the step that will need most adjusting for different vascular beds or cell types not presented here, is the digestion of vascular tissue. This step must be optimized for cell health without diminishing cell yield. The specific enzymes used and the duration for digestion are critical to optimizing cell health and yield to be able to adequately perform downstream assays. For the identification of membrane expression of CD36, as detected by flow cytometry in subcutaneous and mesenteric endothelial cells (**Figure 4**), a modified version of the digestion protocol originally designed for patch-clamp electrophysiology[28] was developed. This included an extension of the collagenase I digestion time to 30 min to increase cell yields to better meet the demands of flow cytometry vs. what is required for patch-clamp studies. As this modification may impact cell health to some extent, a cell viability stain was used in the flow cytometry analyses to ensure that only viable endothelial cells were assessed following this protocol (**Figure 3**). It is recommended that studies aimed at isolating cells from vascular tissue should include a marker for cell viability prior to assessing the desired approach.

The described protocol is sufficient to liberate viable endothelial cells for analysis and downstream applications from ≤1 mg arterial samples derived from mice; however, if the arteries of interest were to be isolated from different tissues or organisms (e.g., humans) that would result in significantly different arterial masses, one would have to optimize the digestion enzyme content and the duration of incubation to efficiently isolate the cell population of interest. For some vascular beds that begin with even smaller amounts of starting tissue than the subcutaneous and mesenteric beds presented here (e.g., coronary arteries), pooling of arteries from several mice may be necessary per sample. Indeed, this may be a necessary step for any given vascular bed given that the outcome approach requires a relatively high cell yield. A major limitation is that, due to the nature of variations per sample digestion, cell yields can sometimes be significantly different across batches even when from the same vascular bed. This can cause issues when analyzing data and should be accounted for via normalization when appropriate. For instance, CD36 expression was extremely variable in the raw data due to alterations in cell yields across individual samples of subcutaneous and mesenteric adipose arteries. Therefore, raw data was normalized to CD31+CD45− mean fluorescence intensity with the assumption that this method would correct for batch differences (**Figure 4**). Of course, depending on the approach, more sophisticated statistical analyses and normalization methods may be required.

In summary, this paper presents a method to dissect, isolate, and digest subcutaneous and mesenteric arteries from mice to investigate the expression and/or function of endothelial targets of interest. With modifications to the presented protocol, different vascular beds and cell types (e.g., smooth muscle cells) can be investigated. This protocol, as a foundation for a plethora of available experimental approaches, has the potential to advance the understanding of vascular cell biology and mechanisms of vascular dysfunction.

Disclosures

The authors have no conflicts of interest.

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