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Original Articles

A Simple Method of Isolating Mouse Aortic Endothelial Cells

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In the study of vascular biology, analyses of endothelial cells (EC) and smooth muscle cells (SMC) are very important. The mouse is a critical model for research, however, the isolation of primary EC from murine aorta is considered difficult. Previously reported procedures for the isolation of EC have required magnetic beads, or Fluorescence Activated Cell Sorting (FACS) to purify the cells. In addition, these procedures were applied to the heart, eyeball, or lung, not the aorta. Therefore we developed a simple method of isolating EC or SMC from the murine aorta without the need for any special equipment. To verify the purity of the cell culture, we performed both an immunofluorescence study and a DNA microarray analysis. The immunofluorescence study demonstrated specific expression of PECAM-1 in isolated EC cultures. In contrast, the isolated SMC didn't exhibit PECAM-1, but rather, smooth muscle actin. The DNA microarray analysis demonstrated the expression of EC (16 genes) or SMC (5 genes) specific genes in each cell. This is due to the fact that pure EC or SMC can be isolated from the aorta, without the use of any special equipment. These results suggest that this method should be particularly useful for vascular biological research. *J Atheroscler Thromb, 2005; 12: 138–142.*

Key words: Murine aortic endothelial cell, Murine aortic smooth muscle cell, DNA microarray, *In vitro* culture

Introduction

In recent years, cardiovascular disease has emerged as the leading cause of death in developed countries (1). The formation of atherosclerotic lesions involves the recruitment of blood monocytes to the arterial intima, the engulfment of lipids, and transformation into macrophage foam cells (2, 3). Endothelial cells (EC) are activated during this process. Hence the focus on EC for investigations of vascular biology is important.

At present, human umbilical vein endothelial cells (HUVEC) are most often used in investigations into EC (4). This is because these cells are easy to obtain and culture, and have been demonstrated to yield reproducible data. However, there is an intrinsic problem in that HUVEC are not from arteries but from veins.

In this paper, we report a novel method of isolating EC and SMC from murine aorta. The mouse is critical to basic research because techniques for genetic manipulation are developed more fully for the mouse than for any other mammalian species.

Currently, there have been reports on methods to isolate EC only from the murine heart (5–7), eyeball (8) or lung (5, 7). Moreover, most of these methods require special equipment, such as magnetic beads combined with antibodies (5, 6) or a FACS (9, 10) to remove the contaminating SMC.

The method described here can be used to isolate not only EC but also SMC, and does not require any special equipment. We verified the character of EC or SMC, respectively, using both an immunofluorescence technique and a DNA microarray. This method is also applicable to the study of transgenic mice.

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Materials

Experimental materials and reagents

Mice

C57BL/6J (male and female) mice (8–12 weeks of age) were purchased from Clea Japan Inc. (Tokyo, Japan) and bred. All procedures involving experimental animals were conducted in accordance with protocols approved in the local institutional guidelines for animal care of the Research Center for Advanced Science and Technology, The University of Tokyo.

Antibodies and other reagents

Pentobarbital sodium was obtained from the Dainippon pharmaceutical CO. LTD. (Osaka, Japan); twisted silk from Natsume (Tokyo, Japan); the catheter (24-gauge 3/4") from the Terumo corporation (Tokyo, Japan); Dulbecco's modified eagle's medium (DMEM), penicillin-streptomycin, L-glutamine, non-essential amino acids and sodium pyruvate from GIBCO BRL, Invitrogen Life Technologies (Carlsbad, CA, USA); and fetal bovine serum (FBS), HEPES, Endothelial cell growth supplements (ECGS), heparin, Dulbecco's phosphate buffered saline (PBS), saponin and bovine serum albumin (BSA) from Sigma (St. Louis, MO, USA). FBS for all experiments was used after it was heat-inactivated. Collagenase type II was obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA); Purified rat anti-mouse CD31 (platelet endothelial cell adhesion molecule: PECAM-1) monoclonal antibody from Pharmingen (San Jose, CA, USA); Rabbit polyclonal smooth muscle actin antibody from Abcam Limited (Cambridgeshire, UK); secondary antibodies (Alexa Fluor 488 donkey anti-rat IgG and Alexa 594 donkey ant-goat IgG) and antifade reagent from Molecular Probes (Invitrogen); mouse pancreas microvascular endothelial cells (MS1) from ATCC (Manassas, VA, USA); and ISOGEN from the Nippon Gene Co. Ltd. (Tokyo, Japan) . The affinity resin column (Rneasy) was obtained from Qiagen (Valencia, CA, USA); collagen Type I- and gelatin-coated dishes from the Asahi Techno Glass Corporation Scitech Division (Tokyo, Japan); and Cover slips and slide glasses from Matsunami Glass INC., LTD (Osaka, Japan). The Leica MZ125 stereoscopic microscope and the Leica DMLB Immunofluorescence observation device were obtained from Leica Microsystems AG (Schweiz, Germany).

Methods

Isolation of EC

See Fig. 1.

1. Two male or female mice are anesthetized with an intraperitoneal injection of 0.3–0.4 ml of pentobarbital sodium (10 mg/ml) per mouse.
2. The midline of the abdomen is incised, and the thorax opened to expose the heart and lungs.
3. The abdominal aorta is cut at the middle to release the blood, and then perfused with 1 ml of PBS containing 1,000 U/ml of heparin from the left ventricle.
4. The aorta is dissected out from the aortic arch to the abdominal aorta, and immersed in 20% FBS-DMEM containing 1,000 U/ml of heparin.
5. The fat or connecting tissue is rapidly removed with fine forceps under a stereoscopic microscope.
6. A 24- gauge cannula is inserted into the proximal portion of the aorta. After ligation at the site with a silk thread, the inside of the lumen is briefly washed with serum-free DMEM.
7. The other side is bound and filled with collagenase type II solution (2 mg/ml, dissolved in serum-free DMEM). After incubation for 45 min at 37°C incubation, EC are removed from the aorta by flushing with 5 ml of DMEM containing 20% FBS.
8. EC should be collected by centrifugation at 1,200 rpm for 5 minutes. Then the precipitate is gently resuspended by pipette with 2 ml of 20% FBS- DMEM and cultured in a 35 mm collagen Type I-coated dish.
9. To remove SMC, after 2 h incubation at 37°C, the medium is removed, the cells are washed with warmed PBS, and medium G (20% FBS, 100 U/ml penicillin- G, 100 µg/ml streptomycin, 2 mM L-Glutamine, 1 × non- essential amino acids, 1 × sodium pyruvate, 25 mM HEPES (pH 7.0–7.6), 100 µg/ml heparin, 100 µg/ml ECGS, and DMEM) is added (7). One week later, confluent EC are observable.

Isolation of SMC

See Fig. 1.

1. Steps #1–#8 are the same as above for the isolation of EC.

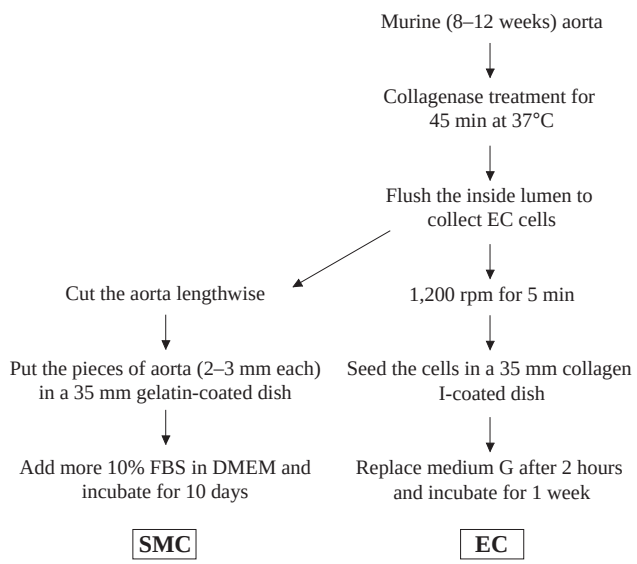

Fig. 1. The method for collecting cells.

2. The blood vessel is cut lengthwise, and put inside of the aorta onto a 60 mm gelatin dish.
3. With a scalpel, the aorta is cut into pieces that are approximately square and 2–3 mm on each side.
4. The SMC are allowed to dry briefly for one minute, 10% FBS in DMEM is added gently, and the cells are placed in an incubator, and left undisturbed for approximately 10 days.

Characterization of EC and SMC

Cells were fixed with cold methanol for 10min at –20°C on a cover slip (18 mm × 18 mm) in a 6 well plate. After incubation with blocking buffer (1% BSA, 0.1% saponin, and 1% FBS in PBS) for 30 min at room temperature, 2.5 µg/ml of antibody against PECAM-1 and 0.5 µg/ml of antibody against smooth muscle actin were incubated with the cells overnight at 4°C or for 3 h at room temperature. After three washes with washing buffer (0.1% BSA and 0.1% saponin in PBS), cells were incubated with Alexa 488 anti-Rat and Alexa 594 anti-Goat IgG antibodies (1 : 300) at room temperature for 30min. Cells were washed three more times with washing buffer, mounted with antifade reagent, and photographed using a microscope equipped with a digital camera.

RNA extraction and gene chip analysis

When the cells reached 80% confluence, we extracted total RNA with ISOGEN following the manufacturer's instructions. RNA quality was determined by measurement of absorbance at 260 nm, and RNA integrity was checked by formaldehyde gel electrophoresis. EC total RNA was analyzed as described previously (11). Briefly, 5 µg of total RNA was used to generate first-strand cDNA. After second-strand synthesis, biotinylated and amplified RNA were purified with Rneasy and quantitated by spectrophotometer. Affymetrix (Santa Clara, CA, USA) mouse MOE430 Arrays were used in this study. This array contains probe sets for 45,000 transcripts and EST clones. After hybridization, the microarray was washed, scanned, and analyzed with GeneChip Analysis Software (version 4.0, Affymetrix). These data were imported into Microsoft Excel for downstream analysis. Subsequent further analysis (clustering) was carried out with a free software program, Cluster 3.0 (Michel Eisen, Stanford University, http://rana.lbl.gov/EisenSoftware.htm) and Java TreeView Version: 0. 9.5, to compare the gene expression of EC or SMC. The gene's expression is represented by color intensity in the corresponding sample. The brighter the red, the higher the genes' expression value. Black indicates that the value is effectively null.

Results

Immunofluorescence analysis

Figure 2 shows that EC grew with the characteristic 'cobblestone morphology'. SMC grew in a 'spindle-shaped' pattern. We used EC or SMC that had been passaged up to three times. To confirm that the isolated cells were EC or SMC, we carried out double staining using PECAM-1, a specific marker for EC, and Rabbit polyclonal smooth muscle actin antibody, for SMC. Figure 3 shows that EC and MS 1 (a mouse endothelial cell line: positive control) were stained by only PECAM-1. On the other hand, SMC clearly expressed only smooth muscle actin.

Characterization of EC and SMC

Furthermore, each cell type's character was profiled

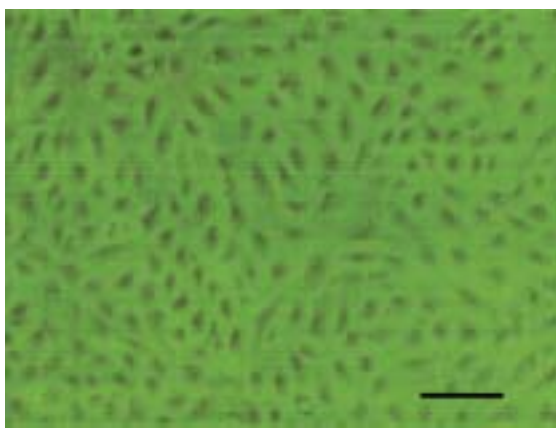

Fig. 2. Morphology of mouse aortic endothelial cells (MAEC). Observation by microscopy (LEICA DM IRB) at 100 × magnification. The bar represents 50 µm.

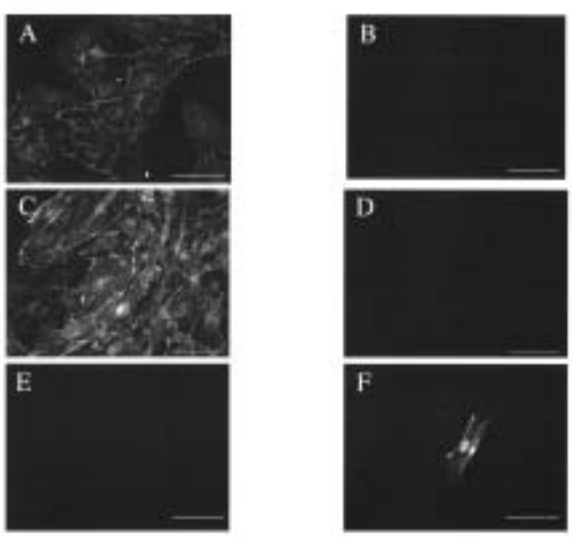

Fig. 3. Immunocytochemistry of murine cells. These cells were immunostained with PECAM-1 (A, C and E) and smooth muscle actin (B, D and F) antibodies. Panels A and B were MS1, Panels C and D were C57BL/6 mouse aortic EC, and Panels E and F were SMC (400 × magnification).

using a DNA microarray (Table 1, Fig. 4). There were a number of EC-specific genes, such as fms-like tyrosine kinase 1 (flt-1), von Willbrand factor (homolog), Cadherin 5 (VE-cadherin), PECAM-1, Ephrin B2, and Intercellular adhesion molecule-2 (ICAM-2), on the list for EC. Smooth muscle actin, calponin-1, and myosin can be seen on the list for SMC. These results also support this method as capable of isolating pure EC or SMC.

Discussion

We have developed a system to isolate and culture EC and SMC from murine aorta without any special equipment. The character of EC or SMC was confirmed both immunohistochemically and by DNA microarray analysis. Both cells could be passaged at least three times.

A method of isolating SMC from the murine aorta has already been established (12), and several different ways to isolate mouse EC have been reported. However, these methods are complicated, and the cells are taken not from the aorta but from the heart, eyeball or lung. Indeed, the use of FACS or magnetic beads based on monoclonal antibodies to remove contaminating SMC or other cells is a useful technique, but unfortunately we have not been able to isolate EC from murine aorta using magnetic beads. Therefore, we have developed a new system to isolate EC from the aorta because such cells are more directly appropriate to the study of atherosclerosis than EC from other organs.

The method described here is quite simple, and can be used to isolate and culture EC or SMC, or even both simultaneously. There are four critical points in the execution of this method. First, before cardiac arrest, the vascular lumen is flushed with PBS (step 3) to minimize the activation of EC due to clots in the lumen. Second is the replacement of the medium after collagenase treatment

Table 1. The principal expressed genes of specific EC and SMC in the isolated EC and SMC.

a. EC specific genes

GenBank*	Name	EC	SMC	EC/SMC
BC020038	endothelial cell-specific molecule 1	244	95	2.6
NM_011171	protein C receptor, endothelial	1,770	69	25.6
NM_010228	fms-like tyrosine kinase 1(flt1)	124	37	3.3
BB133079	endothelial differentiation sphingolipid G-protein-coupled receptor 1	600	40	14.9
NM_008713	nitric oxide synthase 3, endothelial cell (eNos)	1,032	34	30.3
BC024610	vascular endothelial zinc finger 1	255	9	28.0
NM_008816	platelet/endothelial cell adhesion molecule(PECAM-1)	573	18	32.6
NM_011345	selectin, endothelial cell (E-selectin)	89	16	5.5
AF361882	endothelial cell-selective adhesion molecule	796	8	100.7
BB667216	von Willebrand factor homolog	182	1	303.7
AW543698	cadherin 5 (VE-cadherin)	839	6	144.7
NM_010111	Ephrin B2	463	115	4.0
NM_013805	Claudin-5	110	29	3.8
NM_010494	Intercellular adhesion molecule 2 (ICAM-2)	425	1	303.8
NM_010612	Kinase insert domain protein receptor (Flk-1/KDR)	131	41	3.2
NM_013690	endothelial-specific receptor tyrosine kinase	292	11	26.5

b. SMC specific genes

GenBank*	Name	EC	SMC	SMC/EC
NM_007392	actin, alpha 2, smooth muscle, aorta	700	6,559	9.4
AW537707	actin, beta, cytoplasmic	2,089	5,286	2.5
AK007972	myosin, light polypeptide 9, regulatory	29	1,739	59.3
NM_009610	actin, gamma 2, smooth muscle, enteric	7	84	11.9
NM_009922	calponin 1	1	108	90.2

*: GenBank accession numbers for access to the mRNA sequence or spotted cDNA fragment.

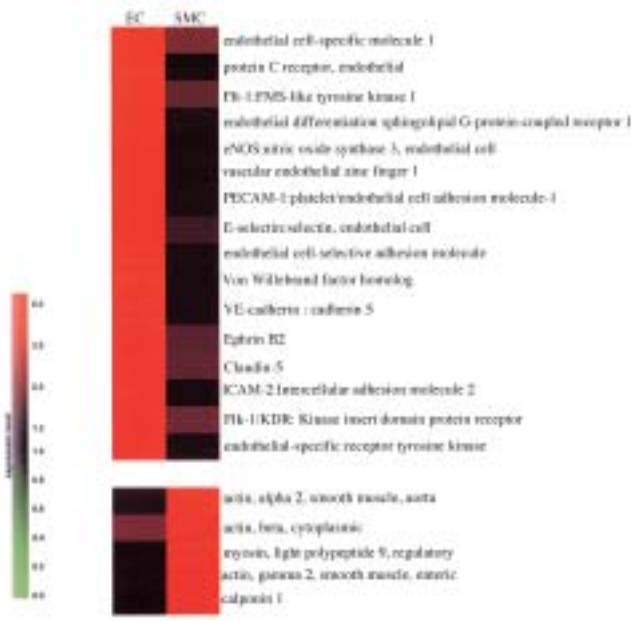

Fig. 4. The hierarchical clustering of mouse aortic endothelial (day 7) and smooth muscle (passage 3) cells. The expression patterns of certain specific genes of EC and SMC were monitored with a hierarchical clustering computer program (GeneSpring).

(step 9). EC attach to the bottom of the plate more quickly than SMC. This step helps to reduce contamination by SMC. If contamination persists, the incubation period may be reduced to 60min or even 30min at step 9. The third critical point is medium G. It contains a large amount of ECGS. In comparison with other reports (5–8), twice as much is contained. This makes EC grow much faster. Even if SMC are present, medium G makes the SMC weaker. The fourth point is the collagen I coat. EC prefer the collagen I coat. The speed of growth is faster and the appearance is better with collagen I than when EC are grown in gelatin-coated or non-coated plastic plates.

We believe that this method of isolating EC or SMC from murine (wild or transgenic) aorta is highly useful for the elucidation of the molecular and cellular mechanisms of cardiovascular disease.

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