

Isolation and Characterization of Human Coronary Artery-Derived Endothelial Cells in vivo from Patients Undergoing Percutaneous Coronary Interventions

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Key Words

Coronary artery disease • Vascular endothelium •
Cell isolation

Abstract

Background/Aims: Human coronary artery-derived endothelial cells (ECs) seem to be the most appropriate cells for the pathogenesis study of coronary artery disease. But limited availability of endothelial tissue is a major constraint. In this study, we developed a method to isolate human coronary artery ECs in vivo from patients. **Methods:** Coronary guidewires were used to obtain EC samples from coronary arteries in 76 patients. Cells were eluted from wire tips and purified by immunomagnetic beads. Von Willebrand factor and CD31 were used as immunocytochemical markers to identify cells as endothelium. Cell viability was evaluated in terms of cell membrane integrity, energy-dependent uptake of Dil-labeled acetylated low-density lipoprotein, and apoptosis. Nitric oxide synthase (eNOS) expression and nitric oxide (NO) production of cells were detected to evaluate cell function. **Results:** About 96 coronary artery ECs were obtained per guidewire. Cells manifested endothelial morphology and immunoreactivity for von Willebrand factor and CD31 with good viability. But eNOS expression and NO

production of cells were decreased. **Conclusions:** Viable coronary endothelium could be obtained during routine percutaneous coronary interventions combined with immunomagnetic beads. These cells may be used for advanced cellular functional analyses such as immunocytochemistry and molecular biology. Such information could aid in understanding mechanisms of coronary artery diseases.

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Introduction

The endothelial cells (ECs) form the inner cellular lining of blood vessels. They are not inert but rather highly metabolically active. Over the last several decades investigators have demonstrated pathogenic roles of endothelial dysfunction in the development of coronary artery disease and other cardiovascular diseases [1, 2]. Yet laboratory experiments almost always use ECs from large vessels such as the human umbilical vein or the bovine dorsal aorta, since these are easy to obtain and can be readily isolated and grown in culture. But it is important to point out that there is marked phenotypic variation between ECs in different parts of the vascular system, such that cells from different locations in the same person not

only express different surface antigens and receptors but can generate different responses to the same stimulus [3–5]. It is also important to note that responses of cultured ECs may not reflect responses seen in the same cells in vivo, and the immortalized EC lines used in many laboratory studies may, in particular, have altered expression patterns of key markers compared with cells studied in vivo. Aside from the organ-specific differences among ECs, human umbilical vein endothelial cells are derived from a vascular bed that does not exist in adults. Therefore, it would seem more appropriate to use coronary ECs isolated from the affected coronary artery bed for its molecular events contributing to the disease rather than the nonspecific analysis of the human umbilical vein endothelial cells under the local pathological environments. Isolation of the ECs from human coronary artery to permit the study of the molecular and/or genetic abnormalities potentially involved in coronary artery disease, however, has not yet been described. In this study, we isolated and characterized human coronary artery ECs in vivo from patients undergoing percutaneous coronary interventions. These findings could facilitate the study of the potential roles of regional ECs in human coronary artery disease.

Materials and Methods

Patient Population

A total of 76 patients (49 male patients, 27 female patients; mean age 65.4 years) with coronary artery disease who presented for percutaneous coronary interventions were included in this study. This study was approved by the Local Ethics Committee, and all patients signed an informed consent that included permission for the use of cells removed during the procedure for subsequent research/educational purposes.

Isolation of Human Coronary Artery-Derived ECs

Immediately after percutaneous coronary interventions, the used coronary guidewires were collected. Tips of the wires (the distal 10 cm) were transferred to EC dissociation buffer (0.5% bovine serum albumin, 2 mmol/l EDTA, and 100 µg/ml heparin in phosphate-buffered saline). Cells were then rinsed in the buffer solution from the wire tips. Immunomagnetic beads (Jingmei Biotech Co. Ltd, China) coated with anti-CD146 antibody (Santa Cruz, USA) were used to purify the ECs. After five washes with DMEM, these cells were ready for the following experiments.

Cell Counting

The purified cells were resuspended in 50 µl of dissociation solution. A smear of 5 µl of the cell suspension was stained with Wright stain solution. The yield of ECs was estimated by counting cells at light microscopy.

Fluorescence Immunohistochemical Study

Immunohistochemical methods were used to characterize the phenotype of human coronary artery-derived endothelial cells (hCADECs). Briefly, cells isolated by immunomagnetic beads were fixed in 4% paraformaldehyde for 15 min, then transferred to slides and air dried. Cells on slides were rehydrated and rendered permeable with 0.1% Triton X-100 for 20 min. The cells were incubated with 1:100 dilution of polyclonal anti-von Willebrand factor antibody (Zhongshan Golden Bridge Biotech Co. Ltd, China) for 1 h at 4°C, followed by secondary antibodies preincubated with fluorescein isothiocyanate (FITC; Zhongshan Golden Bridge Biotech). At the same time, the cells were also incubated with 1:100 dilution of mouse anti-human CD31 antibody (Santa Cruz) and followed by secondary antibodies preincubated with rhodamine (TRITC; Zhongshan Golden Bridge Biotech). Control cells were incubated with washing buffer in place of primary antibody. A fluorescence microscope (Leica, Germany) was used for the examination.

Assays for EC Viability

Three independent assays were used to evaluate the viability of hCADECs. A simultaneous double-staining procedure using fluorescein diacetate (FDA; Sigma, USA) and propidium iodide (PI; Sigma) was performed to assess the membrane integrity of ECs in the first assay [6]. FDA, a nonpolar ester, passes through cell membranes and is hydrolyzed by intracellular esterases to produce fluorescein. Fluorescein is a polar compound. It passes slowly through a living cell membrane, accumulates inside the cell and exhibits green fluorescence when excited by blue light. While FDA stains viable cells exclusively, injured and dead cells are stained by PI. PI passes through damaged cell membranes and intercalates with DNA and double-stranded RNA to form a bright red fluorescent complex seen in the nuclei of dead cells. Since the dye is excluded by intact cell membranes, PI is an effective stain to identify nonviable cells. The hCADECs were resuspended in 200 µl of DMEM medium which contained 0.25 mg/ml FDA and 5 µg/ml PI and were incubated in a well of an eight-well chamber slide for 30 min. The live and dead cells were determined with the aid of a fluorescent microscope (Leica).

The second assay, the uptake of fluorescent DiI-labeled acetylated low-density lipoprotein was performed to test the metabolic function of ECs. This process is energy-dependent, and only live cells with metabolic activity have this ability. After isolation, the cells were suspended in 200 µl of DMEM medium that contained 10 mmol/l DiI-labeled acetylated low-density lipoprotein (Molecular Probes, USA) and were plated on eight-well chamber slides. Cells were incubated for 4 h before fixation in 4% paraformaldehyde and then observed at a fluorescent microscope (Leica).

In the third assay, apoptosis in hCADECs cells was detected by FITC-labeled annexin V and PI. FITC-labeled annexin V detects phosphatidylserine translocation on the cell surface, a hallmark of early apoptosis. Late apoptosis and necrosis show additional positive nuclear staining with PI. After staining, apoptosis was determined by using fluorescence microscopy.

Investigation of EC Function

The endothelial nitric oxide synthase (eNOS) expression and nitric oxide (NO) production of cells were detected to evaluate the hCADECs function. The ECs from iliac artery without any path-

ological lesions of the same patients were obtained by guidewires at the same time and served as control. The expression of eNOS was assayed by the quantitative immunofluorescence method. The eNOS immunohistochemical stain procedure was similar to the von Willebrand factor described above, except that the first antibody was anti-eNOS (Santa Cruz). Staining was visualized under a fluorescent microscope. Images were saved and the fluorescent intensity of eNOS was analyzed by using software Image-Pro Plus. The background of cell images was optimized and non-specific extracellular signal was reduced to a uniform black. The expression of eNOS was quantified by determining average fluorescent intensity of positive (bright) intracellular pixels. At least 30 ECs were analyzed from each guidewire.

Nitrite accumulation, an indicator of NO production of cells, was measured by NO assay kit (Biotech Institute of Nanjing Jiancheng, China), following the manufacturer's instruction. Briefly, cell samples were homogenized in phosphate-buffered saline solution (pH 7.4) and centrifuged at 10,000 g at 4°C. The supernatants were used for determination of nitrate/nitrite by a modification of Griess reaction [7]. Nitrate in the samples was first converted into nitrite with nitrate reductase. After an incubation of 45 min, the samples were mixed with a solution consisting of equal volumes of 14 mM dapsone (4,4'-diamino-diphenyl sulfone) and 4 mM N-(1-naphthyl)ethylenediamine. The color was allowed to develop for 5 min, and absorbance was read at 550 nm by a spectrophotometer. Nitrite concentrations were calculated by comparison with the absorbance of standard solutions of sodium nitrite. For normalization of the measurement, the cell number of each sample was counted under an inverted phase contrast microscope before homogenization. The NO production of cells was normalized as nitrite concentration per 100 cells. NO is a gaseous free radical with a short half-life in vivo of a few seconds or less. It may be quickly oxidized to nitrite and nitrate. In this assay, the levels of the stable NO metabolites, nitrite and nitrate, were used in the indirect measurement of NO. After homogenization, both intracellular and extracellular nitrate/nitrite was collected in the supernatants. With enzymatic conversion by nitrate reductase, nitrate was converted to nitrite and total nitrite was measured, providing a comprehensive overview of endothelial NO formation.

Cell Culture

Cells purified by immunomagnetic beads were resuspended in DMEM medium containing 20% FBS (Gibco, USA), 2 mM L-glutamine, 20 mM HEPES, 100 µg/ml streptomycin, 100 U/ml penicillin, freshly added heparin at 55 U/ml, and endothelial growth supplements 100 µg/ml (ECGS; Sigma). Cells were then plated into a single well of a 96-well plate and incubated at 37°C with 5% CO₂. Culture medium was changed the following day, and then every other day. Cells were confirmed by immunohistochemical staining with von Willebrand factor.

Detection of Circulating ECs Adhering to the Tip of Guidewire

An additional guidewire was used to detect whether circulating ECs which were present in the blood might be harvested together with hCADECs. In brief, two coronary guidewires were inserted into the catheter in the same patient. One wire was used for the subsequent coronary intervention and for hCADECs collection. The other wire was pushed forward just until the tip reached the outlet of the catheter under fluoroscopic guidance,

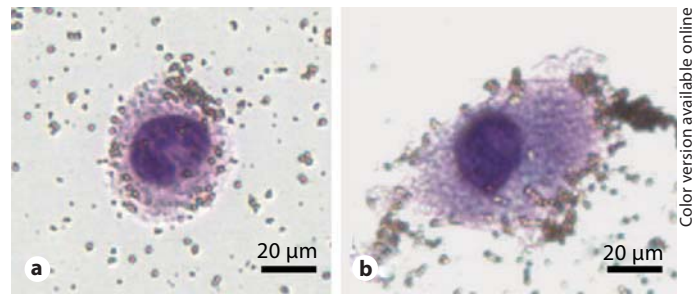


Fig. 1. Wright staining of hCADECs purified by immunomagnetic beads. Cells displayed a round morphology (a) or an oval morphology (b). Particles around the cells were immunomagnetic beads (about $1.00 \pm 0.05 \mu\text{m}$ in diameter).

and was kept in the catheter without any contact with the vessel wall. The cells attached to this wire represent circulating ECs. Cell samples obtained from each wire tip were separately harvested by immunomagnetic beads immediately after use. Circulating ECs were also identified by von Willebrand factor and CD31.

Statistical Analysis

All values were expressed as means \pm SEM. Student's t test was used to determine the statistical differences between hCADECs and iliac artery ECs in all measurements. Differences were considered statistically significant at $p < 0.05$.

Results

Isolation and Characterization of hCADECs

Seventy-six coronary guidewires were collected to sample hCADECs in the selected 76 patients. Of the 76 endothelial samples, 15 were used for Wright staining and cell number counting, 12 were used for fluorescence immunohistochemical study to characterize the phenotype of hCADECs, 5 were used for FDA-PI simultaneous double staining to assess the membrane integrity, 5 were used for the uptake of fluorescent DiI-labeled acetylated low-density lipoprotein to test the metabolic function, 5 were used for annexin V-PI staining to detect cell apoptosis, 12 were used for the investigation of eNOS expression, 15 were used for the measurement of intracellular NO production, and 7 were used for cell culture.

After Wright staining, hCADECs isolated by immunomagnetic beads displayed a round or oval morphology, more than 20 µm in diameter, with a granular pink cytoplasm and an oval red-purple nucleus. Immunomagnetic beads bound to the cells could be clearly seen (fig. 1a, b). The cell count per slide averaged 9.6 (range of 3–14,

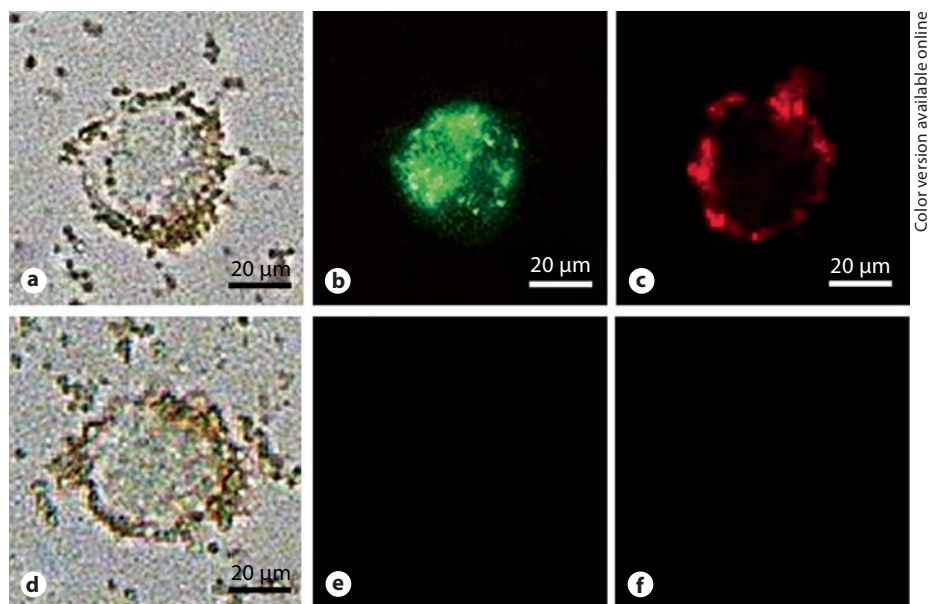


Fig. 2. Immunofluorescent characterization of hCADECs. **a** Bright-field photomicrograph of an hCADEC. **b, c** Cell (**a**) was double stained with von Willebrand factor and CD31. The expression of von Willebrand factor in cytoplasm (**b**) and CD31 on membrane (**c**) is shown. **d-f** A control hCADEC which was incubated without primary antibodies to prove the specificity of staining.

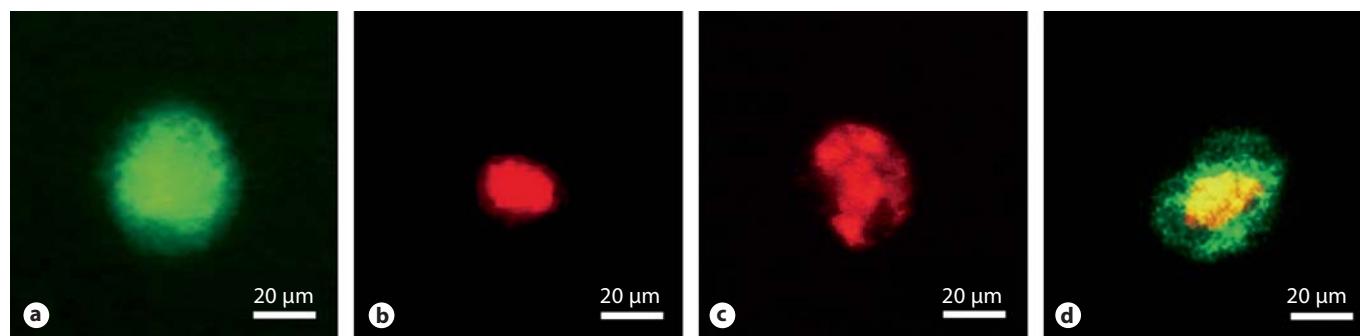


Fig. 3. Assays for EC viability. **a, b** FDA-PI double staining of two different hCADECs for assay of membrane integrity. Live cells appeared green because of the uptake of FDA. Dead cells appeared red because of the uptake of PI. **c** hCADECs after uptake of DiI-labeled acetylated low-density lipoprotein for the assay of metabolic function. **d** Detection of apoptosis hCADECs cells by FITC-labeled annexin V and PI. This cell exhibited a late apoptotic (or necrotic) labeling pattern (annexin V-positive and PI-positive).

estimated total of 96 ECs per subject) in the samples collected from 15 randomly selected patients.

Anti-CD146 antibody was quite specific for ECs [8]. This may ensure the accurate isolation of hCADECs with anti-CD146 antibody-coated immunomagnetic beads. To further confirm their endothelial phenotype we also used fluorescence immunohistochemical staining of harvested cells. 97.6% of the collected cells were positive for von Willebrand factor, 95.8% were positive for CD31, and 94.5% were double positive (fig. 2b, c).

Cell Viability

FDA-PI double staining was performed in 5 hCADEC samples. Viable cells fluoresced bright green, while non-viable cells showed bright red nuclei (fig. 3a, b). Viable hCADECs also had the ability to uptake DiI-labeled acetylated low-density lipoprotein (fig. 3c). Of the total hCADECs from the 10 samples used to evaluate the cell viability, about 96% showed good membrane integrity and metabolic activity.

After being stained by FITC-labeled annexin V and PI in another 5 hCADEC samples, a small number of cells

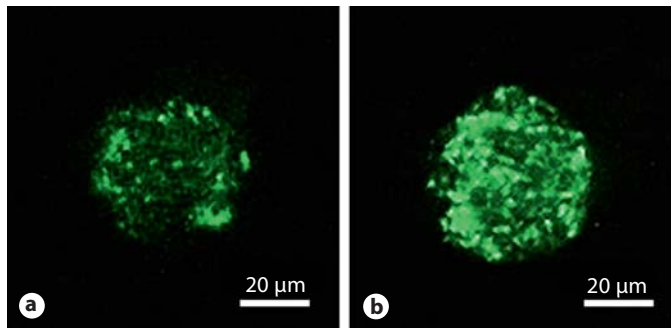


Fig. 4. Immunohistochemical staining for eNOS in two hCADECs. **a** hCADEC showed lower expression of eNOS. **b** EC from iliac artery without any pathological lesions of the same patient showed higher expression of eNOS.

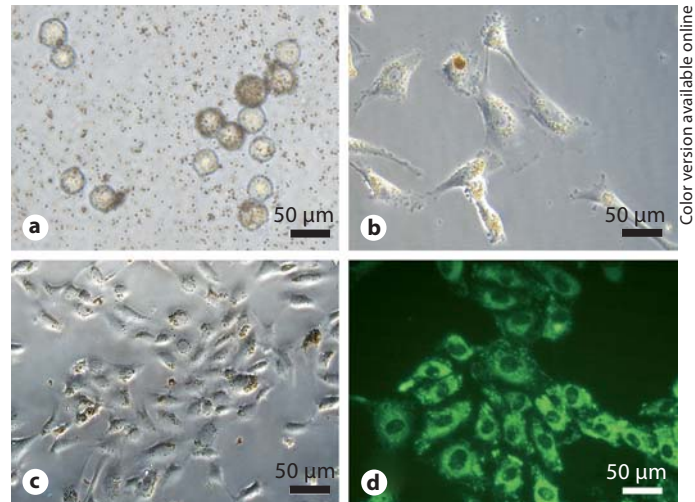
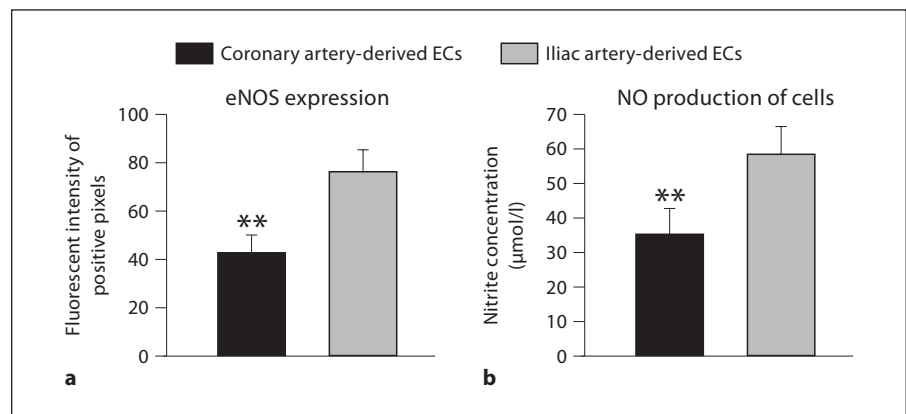


Fig. 6. Culture of hCADECs. **a** Cells immediately after having been plated into the well of a 96-well plate. **b** Cells after 2 days of culture, changing into cobblestone morphology. **c** Semiconfluent monolayer of hCADECs after 7–9 days of culture. **d** The cells cultured showed positive staining for von Willebrand factor.

Fig. 5. Disease-linked dysfunction of hCADECs. **a** eNOS expression. After immunohistochemical staining, the expression of eNOS was quantified by determining average fluorescent intensity of positive intracellular pixels. **b** NO production of cells. Cell samples were homogenized and centrifuged. The supernatants were collected for NO assay by a spectrophotometer. The NO production of cells was normalized as nitrite concentration per 100 cells. ** $p < 0.01$ versus ECs from iliac artery without any pathological lesions of the same patient.



exhibited an early apoptotic labeling pattern (annexin V-positive but PI-negative, about 7%) as well as a late apoptotic (or necrotic) labeling pattern (annexin V-positive and PI-positive, about 2%, fig. 3d).

EC Function

The expression eNOS was visualized under a fluorescent microscope (fig. 4a, b) and quantified as average fluorescent intensity of positive intracellular pixels. Compared with the ECs from iliac artery without any pathological lesions, the eNOS expression in hCADECs of the

same patient was significantly lower (42.4 ± 7.6 vs. 76.2 ± 9.1 , $p < 0.01$; fig. 5a). NO production of cells was also decreased (35.2 ± 7.6 vs. 58.4 ± 8.1 $\mu\text{mol/l}$, $p < 0.01$; fig. 5b).

Cell Culture

hCADECs showed a round morphology with bound immunomagnetic beads after plating in a single well of a 96-well plate (fig. 6a). After 4 h of incubation, the primary cells began to attach. Two days later, these cells changed to a cobblestone morphology (fig. 6b). They started to

proliferate after 3–5 days and became a semiconfluent monolayer after 7–9 days in culture (fig. 6c). After this, most cells began to die gradually. The cultured cells showed positive staining for von Willebrand factor (fig. 6d). The cell number was counted at light microscopy directly after isolation and after cell culturing to estimate the expansion capability. Culture could increase the cell number by about 8-fold.

Detection of Circulating ECs

The circulating EC count per wire averaged 5.4 (range 2–11) in the samples collected from 5 patients. While the number of cells obtained from the wires for hCADECs collection was significantly higher (average 102.6, range 48–153). The circulating EC displayed a similar morphology to hCADECs, and immunohistochemical staining was both positive for von Willebrand factor and CD31 (data not shown).

Discussion

Coronary artery disease is a multifactorial disease, involving hemodynamic, metabolic, and lifestyle determinants that cannot be easily mimicked in experimental animals. Accumulated data have also clarified the tissue-specific or vascular bed-specific characteristics of ECs in the pathogenesis of various disorders [3–5]. The concept of ‘EC heterogeneity’ has been well accepted in vascular biology in recent years. Recognizing the importance of heterogeneity, we should select appropriate ECs to study their potential roles in different disease states. Therefore, hCADECs seem to be the most appropriate cells for the study of pathogenesis of coronary artery disease. But limited availability of endothelial tissue is a major constraint when investigating the cellular mechanisms. Vessel samples may be obtained from patients undergoing coronary artery bypass surgery or heart transplantation or from autopsy. Yet the restricted patient population limits its application. What is more, cells harvested at autopsy may undergo phenotypic drift and cannot reveal their real pathophysiological states *in vivo*.

In this report, we isolated and characterized hCADECs from patients undergoing percutaneous coronary interventions. This study is the first report on the isolation of EC from human coronary artery *in vivo*.

Recently, an effective method has become available for the isolation of ECs from patients’ noncoronary arteries, using vascular guidewires. In 1999 Feng et al. [9] reported a method using guidewires to obtain samples of ECs

from large arteries and veins in patients. They obtained about 262 ECs per guidewire. The most exciting implication of their results was the detection of messenger RNA from obtained ECs by using single-cell reverse transcription PCR. In 2002 Colombo et al. [10] isolated ECs from superficial forearm veins and radial arteries using this method. They reported a reliable measurement of protein expression by quantitative immunofluorescence analysis. They also proved that this analysis was validated against immunoblot analysis in cultured ECs. Feng et al. [11] improved the isolation method in 2005 by using guidewires combined with immunomagnetic beads. This improvement eliminated the possible contamination of smooth muscle cells or blood cells in EC samples. The use of a small number of purified cells as the RT-PCR templates improved the sensitivity and reliability over single-cell RT-PCR. These creative works and the development of coronary interventional devices in recent years provide us with a useful technique to get ECs from human coronary artery.

In this study, we developed a method to obtain human coronary artery endothelium at the vessel bed of vascular lesions. This method of endothelial sampling is safe because it does not prolong the endovascular procedure or involve additional risks for the patients. During endovascular procedures of percutaneous coronary interventions, guidewires inevitably came into contact with the vessel wall. ECs became adherent to the wires. Because the wire was shielded by the catheter from the circulation except for insertion of the coronary artery, highly tissue-specific ECs could be achieved. After having been detached from the wires, these cells were purified by immunomagnetic beads coated with antibody specific for ECs. About 96 coronary artery ECs were obtained by one guidewire per patient. The cells manifested endothelial morphology and immunoreactivity for von Willebrand factor and CD31 with good viability. Further study of protein by quantitative immunofluorescence analysis could be performed, and enough RNA could be obtained from the isolated cells for genomic analysis. This may help to declare the cellular mechanisms of coronary artery disease. The number of cells obtained with the guidewire in our study was less than that of the investigation of Feng et al. [9] and Colombo et al. [10] of large arteries or veins in patients. The thinner and softer guidewires used in coronary intervention may be a possible reason. The powerful heart beating should also be considered.

The isolated hCADECs could be cultured *in vitro* but could not be expanded satisfactorily. The endothelial eNOS expression was assayed by the quantitative im-

munofluorescence method, and the NO production of cells was measured by the NO assay kit to evaluate the hCADECs function. We found that both eNOS expression and NO production of hCADECs were decreased compared with the ECs from iliac artery without any pathological lesions of the same patients. This indicated that these cells were dysfunctional, since they were isolated in patients with severe coronary artery disease. Thus, their proliferation ability would be decreased in this disease state.

Circulating ECs have been found in blood [12] and serve as a sign of vessel wall lesions [13]. These cells may adhere to guidewires and become a potential contamination for hCADECs. But there have been no specific markers to distinguish between these two cell populations until now. To detect the existence of circulating ECs on the wire tip, we used an additional guidewire in the same patient. This guidewire was exposed to the same blood flow as the guidewire used for hCADEC collection, but without any contact with the vessel wall. Therefore, the cells attached to this wire would represent circulating ECs. We found that the number of circulating ECs was considerably smaller than hCADECs. This indicates that the cells harvested by guidewires as hCADECs are mostly derived from the coronary arteries, while a small number of circulating ECs may also be included among them (about 5%). The circulating ECs that adhered to guidewires may consist of two cell populations, already present in blood before coronary intervention and detached from the vessel wall after angioplasty. Circulating ECs are present at a very low frequency in healthy human blood (the concentration usually does not exceed 3 cells/ml) [14]. But in a variety of vascular disorders such as coronary artery disease and after interventional procedures, they are detached from affected vessels and released into blood, and their counts become significantly elevated [15, 16]. As the patients included in our study all suffered from coronary

artery disease and presented for percutaneous coronary interventions, the circulating ECs in blood may perhaps be mainly derived from the affected coronary arteries. Therefore, the ECs on the wires used for hCADECs samples should be mostly derived from coronary arteries. This homogeneity of harvested cells may help to diminish the deviation of subsequent analyses.

This study has several limitations. First, for the safety of patients, the cell isolation procedure was restricted in the population undergoing percutaneous coronary interventions. hCADECs from normal persons or patients with mild coronary lesions cannot be obtained. Second, the number of ECs used for the following RT-PCR analysis is small. Further improvement of the sensitivity and quantification of the RT-PCR analysis with the use of real-time PCR may be more efficient. Finally, the catheter should be modified so that it can be advanced deep into a branch of the coronary artery under fluoroscopic guidance, and that hCADECs can be isolated at sites of vascular lesions.

In summary, we isolated and characterized ECs from human coronary arteries. The cells isolated manifested endothelial morphology and immunoreactivity for von Willebrand factor and CD31 with good viability. This technique can be expanded to isolate ECs with high selectivity from other vessel beds. The cells isolated could provide a source of a primary population of ECs at the disease site for biological analysis. Such information could aid in understanding mechanisms of coronary artery diseases.

Acknowledgments

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