

BRIEF COMMUNICATION

Isolation and Purification of Canine Adipose Microvascular Endothelial Cells

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INTRODUCTION

Genetic engineering and seeding of endothelial cells onto synthetic small-diameter vascular grafts is a promising strategy in improving vascular graft patency (Bellamkonda *et al.*, 1997; Kader *et al.*, 2000; Ratcliffe, 2000). Currently, the source of endothelial cells for vascular graft seeding studies has been large vessels such as the aorta. Endothelial cells derived from large vessels are not a practical source for autologous transplantation, expansion in culture, or genetic modification. We suggest that microvascular cells are appropriate cells for endothelial cell seeding.

We report a protocol designed to increase the purity of canine falciform ligament-derived endothelial cells (CFLEC) suitable for vascular transplantation. Our strategy is based on the use of *Ulex europaeus* agglutinin-1 (UEA-1), an endothelial cell-specific lectin that

binds to the α -1-fucosyl residues of the glycoproteins in humans (Hormia *et al.*, 1983) and other primates (Christenson and Stouffer, 1996). Whereas UEA-1 has been used in the isolation of human microvascular and large-vessel endothelial cells (Conrad-Lapostolle *et al.*, 1996), successful isolation of canine microvascular endothelial cells has not been demonstrated. Our system produces highly pure populations of canine microvascular endothelial cells in approximately 3 weeks.

MATERIALS AND METHODS

Adipose Tissue Collection and Tissue Digestion

Falciform ligament fat was collected from male mongrel dogs, handled under NIH guidelines for the ethical treatment of large animals. A midline incision of 5 cm was made distal of the sternum and 3 to 5 g of falciform ligament was identified and harvested 30 min after sacrifice. The collected falciform ligament tissue was digested using a protocol modified from Chung-Welch *et al.* (1997). A solution of 0.1% clostrid-

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ial collagenase (Worthington Biochemical) and 10 mg/ml porcine heparin (Sigma) in Medium 199 with Hepes salts (Life Technologies) was added to the collected tissue at 1 ml solution/g fat tissue. The tube was incubated at 37° for 30 min. The tissue was washed with 70% ethanol to fix residual mesothelial cells and rinsed with Hank's Balanced Salt Solution (HBSS, Life Technologies). The tissue was placed in a fresh tube of 0.1% collagenase and 10 mg/ml heparin solution. The tissue was finely minced with sterile scissors. The tube was vigorously shaken and incubated at 37° for 30 min with shaking every 10 min to ensure proper mixing.

The homogenate was filtered through sterile gauze to remove any large pieces of undigested tissue. Cells were pelleted by centrifugation at 900g for 10 min. The pellet was washed three times with 10 ml of HBSS containing 10 mg/ml porcine heparin to remove residual blood cells and adipocytes.

Endothelial Cell Labeling and Isolation

The cell pellet was resuspended in 0.5 ml HBSS. Endothelial cells were labeled using fluorescein isothiocyanate (FITC)-tagged UEA-1 added at a concentration of 150 µg/ml of solution. Briefly, the cell suspension was incubated with UEA-1 at 4° for 10 min with intermittent inversion. After incubation, the cell suspension was filtered through a sterile 67-µm nylon filter mesh to remove cell clumps and debris. The concentration and diameter distribution of the filtrate were assayed with a Coulter Multisizer IIe (Beckman Coulter, Inc., Fullerton, CA). Thirty million cells were transferred to a 15-ml centrifuge tube and washed with sterile buffer: 0.5% bovine albumin and 2 mM EDTA in phosphate-buffered saline. One microliter of anti-FITC-MACS magnetic nanospheres (Miltenyi Biotec, Auburn, CA) and 9.0 µl of buffer were added per million cells to the cell pellet. The suspension was mixed, incubated for 30 min at 4°, washed, resuspended in 0.6 ml buffer, and counted. The sample was enriched for cells expressing UEA-1 by magnetic separation on a mini-MACS column (Miltenyi Biotec). Two passages through the mini-MACS columns were used to achieve high purity in the positive fraction. Multisizer counts, gravimetric determination of volume, and flow cytometry analysis of the positive and

negative fractions from the column enabled calculation of positive cell purity and recovery.

Cell Size Characterization

Aliquots of the crude cell isolate and positive and negative fractions of the purified cells (after UEA-1-based separation) were characterized by size distribution. A Coulter Multisizer IIe was used to provide a size distribution before and after purification.

Cell Culture

All isolated canine microvascular cells were cultured at 37° with 5% CO₂ and 95% humidity. Both purified and nonpurified CFLEC were cultured in DMEM (Life Technologies) supplemented with 20% fetal bovine serum (Hyclone), 1 µg/ml penicillin-streptomycin (Life Technologies), 75 µg/ml endothelial cell growth supplement (Upstate Biotech), 1 µg/ml insulin-transferrin-selenium supplement (Sigma), and 100 µg/ml porcine heparin (Sigma).

Morphological Assessment

Purified and nonpurified cells were allowed to attach to human plasma fibronectin (5 µg/cm², Chemicon)-coated tissue culture flasks (Falcon). Assessment of morphology occurred at 3 days and 3 weeks (for purified cells) after the initial digest using a Nikon TE-300 microscope and attached Javelin CCD camera.

Immunohistochemical Assessment

Isolated cells were allowed to attach and reach at least 50% confluence in eight-chamber glass slides (Falcon) and were fixed in Histochoice (Lab Supplies). Identity of cells that had undergone full purification and cells from the crude digest were tested using factor VIII/vWF (rabbit anti-human, Dako) and CD31 antibodies (mouse anti-human, Dako).

RESULTS

Cell Size Characterization

Figure 1 shows the size distribution of the crude collagenase-digested cell fraction (unpurified, Fig. 1A), cells

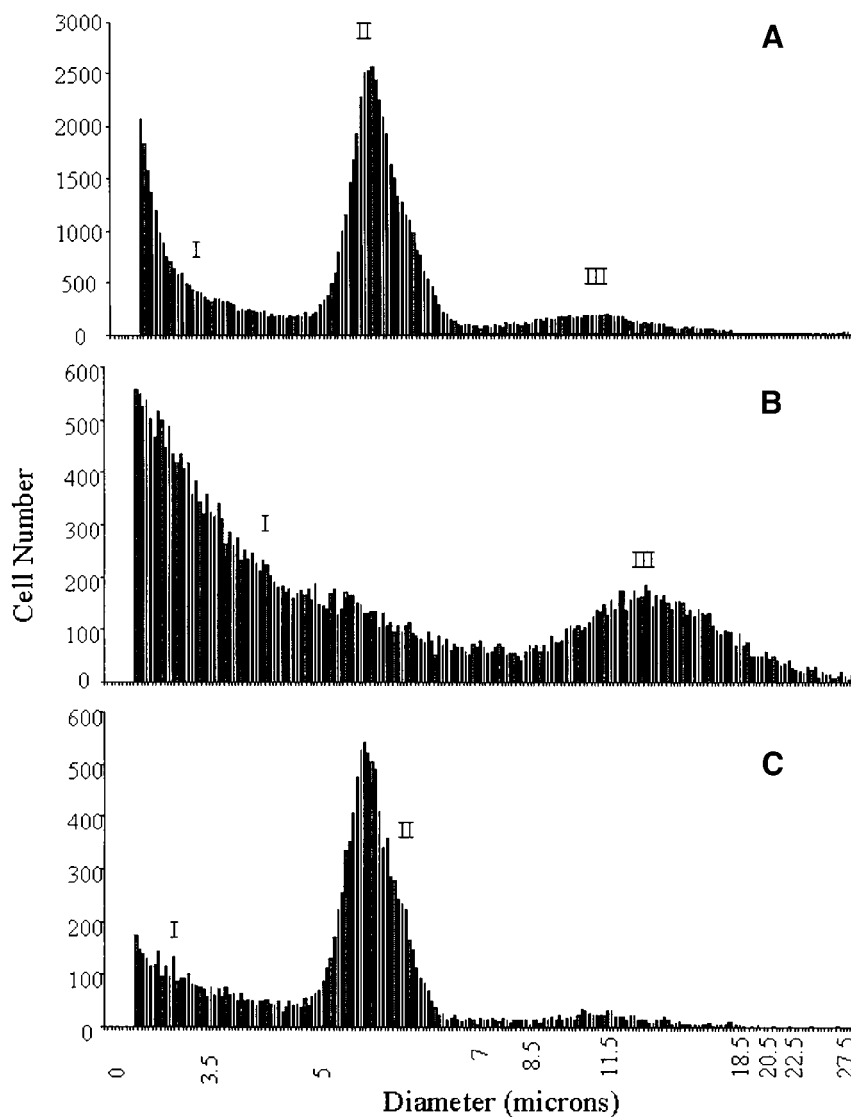


FIG. 1. Size distribution of fractions obtained after use of the isolation protocol. Graphical representation of the size distribution of (A) unpurified microvascular cells, (B) UEA-1 positive cells, and (C) UEA-1 negative cells. (A) Three peaks corresponding to platelets (peak I), pericytes and red and white blood cells (peak II), and endothelial cells (peak III). (B) Enrichment of peak III corresponding to endothelial cells. (C) Enrichment of peak II corresponding to pericytes and red and white blood cells.

positive for UEA-1 (positive, Fig. 1B), and cells negative for UEA-1 (negative, Fig. 1C) as assessed by a Coulter Multisizer IIe. Three primary peaks were discerned in the unpurified fraction: peak I (1.5–3 μm) corresponding to platelets, peak II (3.0–7.5 μm) corresponding to pericytes (Rodriguez-Baeza *et al.*, 1998) and red and white blood cells, and peak III (8–18.5 μm) corresponding to endothelial cells. Decreases in platelets, pericytes, red and white blood cells (peaks I and II) in the positive

fraction, therefore red and white blood cells were seen primarily in the negative fraction. CFLEC (peak III) were seen in the positive fraction.

Cell Culture

UEA-1-purified endothelial cells attained a confluent monolayer after 3 weeks of culture. Figure 2A

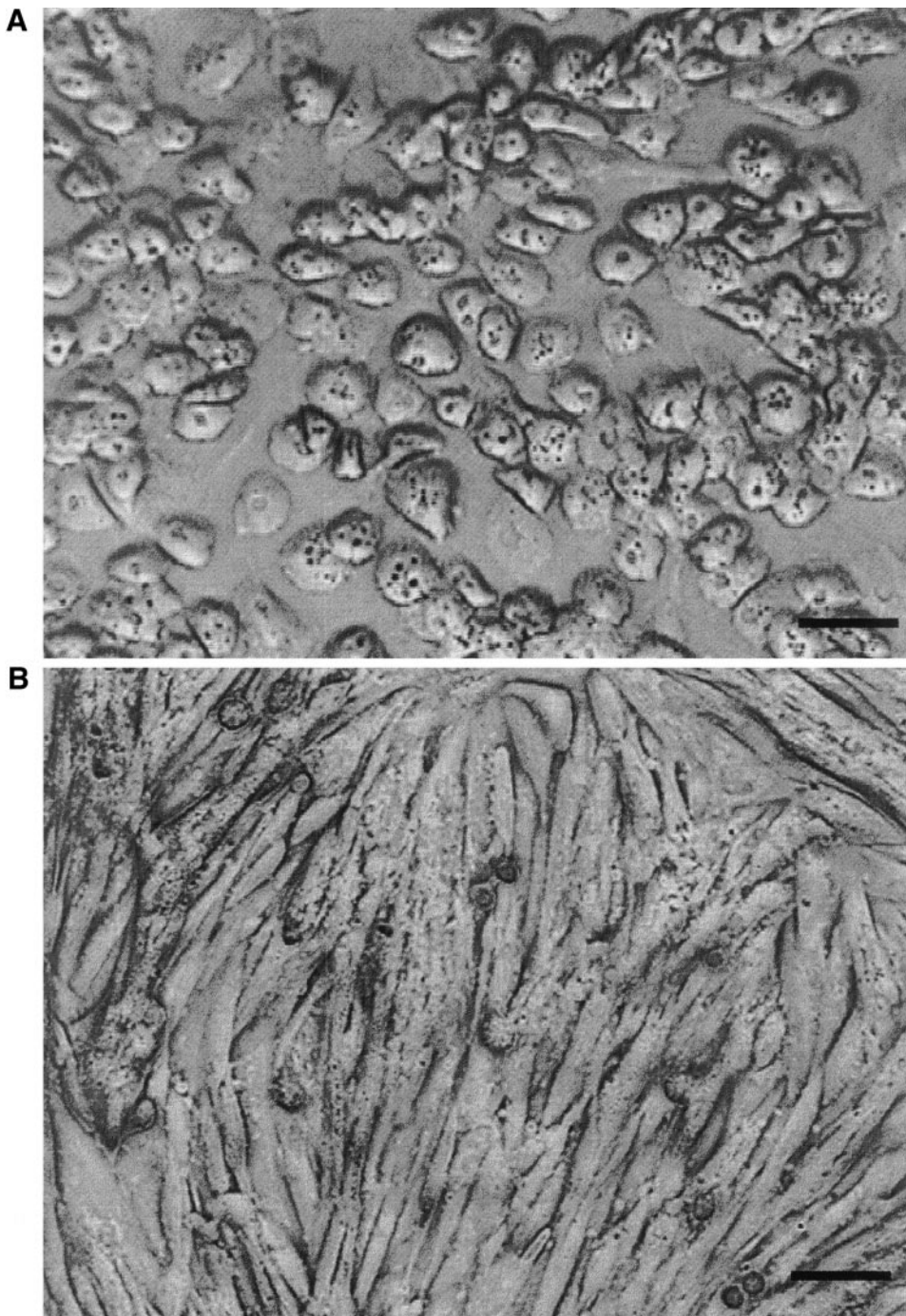


FIG. 2. Primary purified and nonpurified isolates. (A) UEA-1 positive isolate at 3 weeks after isolation. Cells appear endothelial in shape and arrangement on the tissue culture dish. (B) UEA-1 negative cells at 3 days after isolation. The population has clearly changed phenotype and resembles smooth muscle or fibroblast morphology. Scale bar represents 25 μm .

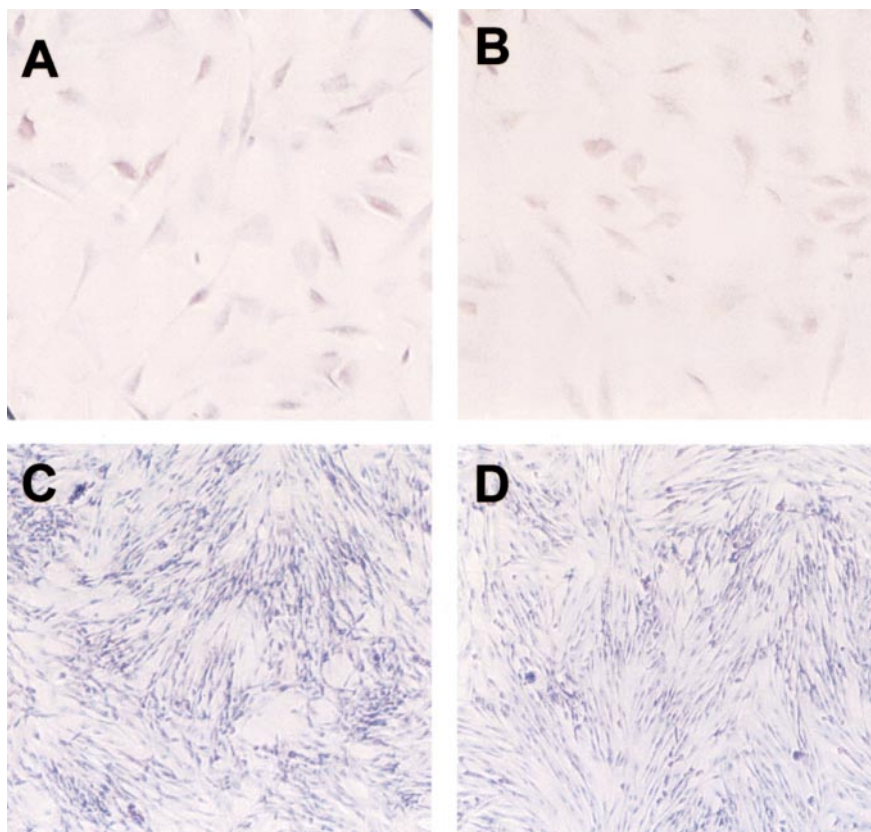


FIG. 3. Immunohistological staining of UEA-1-purified and nonpurified microvascular cells. UEA-1 positive first-pass canine microvascular endothelial cells stain positive for CD31 (A) and Factor VIII (B). Unpurified cells were negative for CD31 (C) and Factor VIII (D) staining. Photomicrographs at 10 \times .

shows purified CFLEC at 3 weeks after tissue digest (10 \times magnification). These endothelial cells were viable through the third passage, at which point they became nonadherent to the FN-coated substrate.

Figure 2B shows a nonpurified isolate at 3 days after isolation (10 \times magnification). This mixed cell population achieved confluence at day 4 and did not exhibit contact inhibition. It is interesting to note that our unpurified cells required a relatively short time to transform into the fibroblastic morphology observed and attain confluence. The purified cells, however, required several weeks to attain confluence but maintained a constant phenotype.

Identity Testing

Morphological assessment. Primary purified isolates showed an elongated morphology consistent

with published reports on microvascular cells (Fig. 2A; Williams, 1999). These cells were viable for three passages. In all cases, these cells were contact inhibited and retained their morphology. Nonpurified cells (Fig. 2B) were not contact inhibited and exhibited a classic smooth muscle cell "hill-and-valley" configuration. These cells were spindled and resembled smooth muscle cell morphology.

Immunohistochemical assessment. Figure 3A shows CD31 and Factor VIII staining of purified endothelial cells. There was positive staining for both CD31 and factor VIII/vWF, indicating typical endothelial cell phenotype. Staining of the nonpurified fraction showed negative staining for both CD31 and Factor VIII (Fig. 3C and 3D, respectively). Staining of both the UEA-1 purified and the nonpurified fractions occurred at 3 weeks after isolation.

DISCUSSION

The canine model of vascular disease is important. However, the procurement and long-term expansion of canine microvascular endothelial cells necessary for many vascular trials has not been well studied. Whereas Williams and colleagues reported the short-term culture of endothelial cells derived from falci-form ligament fat, long-term cultures have not been described.

Present techniques for endothelial cell isolation rely on the relative abundance of this cell type with respect to that of other vascular cells within a crude cell isolate. Whereas endothelial cells are the most significant of the adherent populations within the microvasculature, other cells, most notably pericytes, may have deleterious effects on cultured endothelial cells (Martin *et al.*, 2000; Shepro and Morel, 1993).

Our strategy systematically removes contaminating cells, in particular, platelets and pericytes. These cell types are known to produce TGF- β 1, which have been shown to transform endothelial cells to a fibroblastic phenotype (Arciniegas *et al.*, 1992). The initial digest of the collected tissue was designed to remove the mesothelial cells, which are also abundant in the peritoneum. The secondary digest was the main homogenizing step for the tissue and provides the first useful cell suspension, a step at which others have considered the isolation complete (Williams, 1999; Williams *et al.*, 1989, 1994). However, the pellet, even after successive washes, contained pericytes, smooth muscle cells, fibroblasts, and a large number of circulating blood cells, including platelets.

A high heparin concentration was added to these two digest steps to inhibit release of platelet TGF- β 1 by decreasing platelet activation and preventing adverse downstream effects. This heparinization was a pivotal step to prevent activated platelets from clogging the column and decreasing purity. Isolates without this high heparin concentration were of low purity and changed to the same fibroblastic phenotype seen without the purification steps (Fig. 2). Heparin did not appear to be harmful to isolated CFLEC at this concentration.

The use of UEA-1 for endothelial cell isolation has been reported but its use as described for isolating canine microvascular cells is novel. We believe that the method of isolation of canine microvascular endothelial cells reported represents an advance in the isolation and culture of purified endothelial cells without pericyte contamination. These methods may be particularly useful for isolation and expansion of endothelial cells in the tissue engineering of vascular grafts, heart valves, stents, and other cardiovascular devices.

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