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Isolation and characterization of human gingival microvascular endothelial cells

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Background and Objective: Endothelial cells have a substantial role in maintaining vascular homeostasis, and their dysregulation can contribute to the development of pathology. The plasminogen activators and their inhibitors may, arguably, be the single most important proteolytic system of the endothelium for vascular maintenance by controlling plasminogen activation and other proteolytic cascades that impact on clotting, hemodynamics, angiogenesis and the character of the vascular wall. In chronic periodontal disease, significant changes to the microvasculature occur in association with the severity of the disease. Investigation of the role played by endothelial cells in periodontal health and disease has been limited to *in situ* immunolocalization or to the use of endothelial cells of nongingival origin, such as human umbilical vein endothelial cells. The objective of this research was to establish a replicable protocol for isolating microvascular endothelial cells from the gingiva.

Material and Methods: From inflamed gingiva, isolated cells were characterized by morphology, the expression of factor VIII-related antigen, the expression of UEA-1 ligand, the uptake of acetylated low-density lipoprotein, network formation on Matrigel, and by the expression levels of urokinase plasminogen activator, tissue plasminogen activator, plasminogen activator inhibitor-1 and collagen IV.

Results and Conclusion: Gingival endothelial cells were most readily obtained from inflamed gingival tissues, and these endothelial cells, when isolated by the protocol established herein, demonstrated endothelial characteristics and constitutively secreted plasminogen activators and plasminogen activator inhibitor-1 in culture.

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The vast endothelial microvascular network is thought to play an extremely important role in processes ranging from embryogenesis and development, to cancer progression and diabetes-related pathology, throughout the body (1). Macrovascular complications, such as atherosclerotic coronary artery disease, present with subendothelial lipid and matrix alteration that is attributable, in part, to the endothelial dysfunction (2). Studies investigating the pathogenesis of periodontitis and other gingival lesions have examined the roles played by fibroblasts and keratinocytes through the use of primary gingival cell cultures. However, studies aimed at describing the role played by endothelial cells in the gingiva have been limited to histological methods or to the use of endothelial cells of nongingival origin (3–10).

Endothelial cells from different organs have shown a wide range of heterogeneity, potentially limiting the value of nongingival cells in dental

research (11,12). We have found few reports describing the use of isolated gingival microvascular endothelial cells. The ability to isolate these gingival microvascular endothelial cells is critical to the understanding of their role in gingival health and disease and important for the advancement of oral and periodontal research.

Historically, the isolation of microvascular endothelial cells has been challenging. Contamination by competing fibroblasts, smooth muscle cells and mesothelial cells in culture, coupled with limited sources, low yields and the slow growth of these cells, has made them particularly difficult to isolate (12,13). Methods for isolating endothelial cells have progressed from manual weeding, to selection based on specific markers including lectins such as *Ulex europaeus* lectin type I, acetylated low-density lipoprotein uptake, human leucocyte antigen-DR, Factor VIII-related antigen and platelet-endothelial cell adhesion molecule 1 (12–15). Platelet-endothelial cell adhesion molecule 1 is a molecule that is expressed constitutively on endothelial cells and hemopoietic cells (monocytes, platelets and T lymphocytes) (16,17), which makes it an ideal candidate for establishing long-term cultures of adherent endothelial cells.

Contributing to the challenge, there is no single marker that can alone distinctly identify endothelial cells. Combinations of characteristic, although not definitive, markers such as these are used, together with functional characterizations including a strong uptake of acetylated low-density lipoprotein, a 'cobblestone' morphology at confluence and tube formation on Matrigel (11,12).

We therefore sought to test the effectiveness of standard endothelial cell isolation techniques on gingival biopsies. Endothelial cells of the macrovasculature and microvasculature express extracellular enzymes and inhibitors related to plasminogen activation, which have been shown to be important in angiogenesis, perivascular wall remodeling and the homeostasis of fibrinolysis (1). The expression characteristics of the plasminogen activators urokinase plasminogen activator and tissue plasminogen activator, as well as their inhibitor, plasminogen activator inhibitor 1, were therefore of particular interest during our characterization of gingival endothelial cells. This report should promote investigation into the role of gingival endothelial cells and the gingival microvasculature in the maintenance of health and development of gingival pathologies.

Material and methods

Cells and cell culture sources

Primary gingival excisional tissue biopsies were obtained with informed consent during gingival surgeries performed at the Nova South-eastern University College of Dental Medicine after Institutional Review Board approval of the study. All biopsies were collected during the course of clinically indicated treatment. Adult normal human dermal fibroblasts (HDF) were purchased from Clonetics (NHDF) (Walkersville, MD, USA). Primary human umbilical vascular endothelial cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Primary adult human dermal microvascular endothelial cells were purchased from VEC Technologies (Renseleer, NY, USA). All cells were grown in EGM-2 MV media purchased from BioWhitaker, a division of Clonetics. Clostridial collagenase islet isolation grade type II, 351 U/mg, was purchased from Invitrogen Corp. (Carlsbad, CA, USA). Dispase was purchased from BioWhitaker. Matrigel, a gel of basement membrane constituents from mouse tumor including various growth factors, was purchased from Becton Dickinson Labware (cat. #354234; Bedford, MA, USA). Anti-CD31-coated Dynalbeads of 4.5 micron diameter were purchased from Dynal ASA (product # 111.28; Oslo, Norway). Calcium chloride was purchased from Fisher Scientific (Suwanee, GA, USA).

Sources of lectin, antibody and plasminogen activator standards

Monoclonal antibody specific for Factor VIII-related antigen (Clone F8/86) was purchased from NeoMarkers (Fremont, CA, USA) as a hybridoma media. *U. europaeus* agglutinin I, a lectin that binds specifically to terminal alpha-L-fucosyl residues of glycoproteins presented on the surface of endothelial cells, was purchased as a fluorescein isothiocyanate conjugate from Sigma Aldrich Corp. (St Louis, MO, USA). For immunodetection of urokinase plasminogen activator, a mouse IgG1 anti-urokinase plasminogen activator was used that recognizes the B-chain near the catalytic site on the single-chain urokinase plasminogen activator, the two-chain high-molecular-weight and low-molecular-weight urokinase plasminogen activator, as well as the urokinase plasminogen activator bound to the urokinase plasminogen activator receptor (product #394; American Diagnostica, Stamford, CT, USA). For immunodetection of tissue plasminogen activator, a mouse IgG1 anti-tissue plasminogen activator was used that recognizes single-chain and two-chain tissue plasminogen activators (product #373; American Diagnostica). For immunodetection of plasminogen activator inhibitor 1, a mouse IgG1 anti-plasminogen activator inhibitor was used that is known to recognize free and tissue plasminogen activator-bound plasminogen activator inhibitor 1 (product #3785; American Diagnostica). To detect collagen type IV, mouse IgG1 anti-collagen type IV was used (Clone Col-94; Sigma Aldrich Corp., St Louis, MO, USA). A goat anti-mouse fluorescein isothiocyanate-conjugated secondary antibody, preadsorbed with human serum, was purchased from Sigma Aldrich Chemicals. 4',6-Diamidino-2-phenylindole, a fluorescent nuclear stain, was purchased from Sigma Aldrich Chemical Corp. (St Louis, MO, USA).

For enzyme-linked immunosorbent assay (ELISA) and dot-blot analyses, the standard for quantification of urokinase plasminogen activator was a single-chain urokinase plasminogen activator (product #107; American Diagnostica). For the quantification of tissue plasminogen activator, the standard was a single-chain tissue plasminogen activator (product #111;

American Diagnostica) and for the quantification of plasminogen activator inhibitor 1, a latent human plasminogen activator inhibitor 1 standard was used (product # PAI-L-603A; Molecular Innovations).

Tissue preparation, cell selection and culture

Immediately after surgical excision, discarded gingival tissue samples from patients were placed in a cold phosphate-buffered saline solution. The quality of tissue collected ranged from firm and healthy to inflamed and diseased. One portion of each sample was separated, fixed in buffered formalin, paraffin embedded, sectioned at 5 μm and then stained with hematoxylin and eosin to identify the presence or absence of inflammatory infiltrate by microscopy. The remainder of each sample was washed three times with phosphate-buffered saline, then weighed and placed in a 2.5-cm^2 Petri dish. Samples were carefully minced into 1-mm^2 pieces. The minced pieces were digested overnight at 37°C, 5% CO_2, in a water jacketed incubator (culture incubator) using 200 IU of Clostridial collagenase and 75 IU of Dispase with 2.5 mL of EGM-2 MV medium containing 5% fetal calf serum in a total volume of 4 mL with 3 mM $CaCl_2$ added. Following the digestion period, the remaining tissue remnants were separated from the digestion by gravity in a conical tube for 3 min. From the digest medium, the cells were pelleted by centrifugation at 1000 *g* for 1 min at room temperature. The pellet was resuspended in 4 mL of EGM-2 MV and plated on a T 12.5 tissue culture flask (Falcon; Becton Dickinson) then incubated overnight at 37°C in a culture incubator. The attached cells were then washed once with phosphate-buffered saline and the medium replaced with identical fresh medium, then the cells were incubated until just reaching confluency (≈ 3–4 d), with daily replacement of the medium with identical fresh medium.

The attached cells were detached with trypsin and washed according to a standard protocol, then resuspended in 1 mL of phosphate-buffered saline containing 0.1% bovine serum albumin (phosphate-buffered saline/bovine serum albumin). Approximately 1.6×10^6 magnetic beads, coupled to monoclonal anti-platelet-endothelial cell adhesion molecule 1, were added to the cell suspension.

Binding of endothelial cells by the beads was optimized by gently rotating the suspension for 20 min at 4°C. After this binding period, the bead/cell suspension was divided into two 0.5-mL portions to which 0.5 mL of fresh phosphate-buffered saline/bovine serum albumin was added. The magnet was used to retain the beads with the bound cells while the unbound fraction was removed. After washing the beads in each tube four times with 1 mL of phosphate-buffered saline/bovine serum albumin, the beads and the bound cells in each tube were resuspended in 1 mL of EGM-2 MV growth medium containing 5% fetal calf serum, and each were plated in a 2-cm^2 well of a 24-well plate and incubated at 37°C (passage 1). The medium was changed daily and the cells were passaged and split by trypsinization, when near confluence (≈ 3–7 d), and transferred to successively larger wells and then to flasks.

Cell characterization

Each of the isolated cell cultures was characterized between the third and the sixth passages. Control dermal fibroblasts, dermal microvascular cells and human umbilical vein endothelial cells were also included in the characterizations because they are commercially available and commonly used cell types. Aliquots of 5000 cells were seeded onto gelatin-coated chamber slides (0.7 cm^2/chamber) and cultured for 3 d, then fixed in a cold 0.1 M solution of acetic acid or buffered 3% formaldehyde for 30 min, then washed and blocked with three changes of phosphate-buffered saline containing 0.1% Tween 20 (phosphate-buffered saline/Tween).

For the identification of Factor VIII-related antigen, cells were incubated for 2 h in blocking buffer with a 1 : 64 dilution of primary antibody F8/C6. The chambers were washed three times in blocking buffer, then the cells were incubated for 1 h with secondary goat anti-mouse fluorescein isothiocyanate conjugate in a humidified chamber at 22°C. For UEA-1 staining, cells were incubated in blocking buffer for 2 h with 16 μg/mL of *U. europaeus* agglutinin I conjugated to fluorescein isothiocyanate.

The chambers were then washed four times with blocking buffer, the chambers were removed, then the slides were mounted with an aqueous mounting media under a coverslip. In control cell aliquots, secondary antibody alone was used to assess nonspecific fluorescence. 4',6-Diamidino-2-phenylindole (20 μg/mL) was applied to all cultures in blocking buffer at the time of application of the secondary antibody.

Endothelial branching assays

To assess the isolated endothelial cells for their functional ability to form networks, Matrigel was used as a substrate, at a concentration of 11 mg/mL solublized in Dulbecco's modified Eagle's medium with 10 μg/mL of gentamycin. Forty microlitres of Matrigel was spread across the bottom of a 2-cm^2 well and 2×10^4 cells were seeded. Cultures were incubated at 37°C in a humidified chamber with 5% CO_2, and the resulting networks were photographed.

Acetylated low-density lipoprotein uptake

Live cells were seeded on gelatin-coated chamber slides at a subconfluent density (7000 cells/cm^2) and cultured for 3 d in EGM-2 MV media. Then cultures were washed once with media then incubated for 4 h in media containing 10 μg/mL of an acetylated low-density lipoprotein (Dil-Ac-LDL; Biomedical Technologies, Inc., Stoughton, MA, USA) at 37° in a culture incubator. The cultures were then washed three times with fresh medium then incubated for a further 16 h. The cultures were then washed three times with phosphate-buffered saline and fixed for 30 min in buffered 3% paraformaldehyde. The fixed cells

were then rinsed briefly with double distilled H_2O, 4',6-diamidino-2-phenylindole (20 μg/mL) was applied in phosphate-buffered saline for 10 min, the chambers were removed and the slides were then mounted with aqueous medium under a coverslip. The uptake of Dil-Ac-LDL by cells of the monolayer was visualized by fluorescence microscopy. Human dermal fibroblasts were used as a negative control.

Plasminogen activator, plasminogen activator inhibitor 1 and collagen type IV expression

Primary gingival, dermal, or human umbilical vein endothelial cells were seeded in 96-well microtiter plates at a density of 10,000 cells per well, allowed to attach overnight, then provided with fresh medium. Cultures were incubated for 5 d, photographed under phase-contrast microscopy, then the conditioned medium was removed and stored until required for ELISA.

ELISA measurement of urokinase plasminogen activator, tissue plasminogen activator and plasminogen activator inhibitor 1 in conditioned medium

ELISA to detect the amount of urokinase plasminogen activator, tissue plasminogen activator and plasminogen activator inhibitor 1 accumulation in conditioned medium was performed and analyzed as previously described (18), determining the concentrations by interpolating the sample absorbance at 405 nm with the linear portion of the standard curve using the standards described above. For the sandwich ELISAs, plates were coated with 5 nM goat polyclonal antibodies for urokinase plasminogen activator, tissue plasminogen activator and plasminogen activator inhibitor 1 (American Diagnostica), the sample was applied, then the monoclonal antibodies described above (sources of lectin, antibody and plasminogen activator standards) were utilized as primary antibodies. Finally, secondary horse anti-mouse biotin conjugate (1 μg/mL; Vector Technologies, Inc., Burlingame CA, USA) was applied for 2 h, followed by streptavadin-alkaline phosphatase conjugate and substrate in excess (4-nitrophenyl phosphate). Substrate development was quantified by measuring the color development at 405 nm.

Immunofluorescence

To assess urokinase plasminogen activator, tissue plasminogen activator, plasminogen activator inhibitor 1 and collagen 4 expression in an alternative manner, cells were seeded, as described above, at a subconfluent density (7000 cells/cm^2) in chamber slides coated with a 1% gelatin solution made up in ddH_2O (Becton Dickinson, Franklin Lakes, NJ, USA). The cells were allowed to seed overnight, then the medium was replaced with fresh medium. After 5 d of incubation, cells were fixed in 10% paraformaldehyde for 20 min, then analyzed for expression of plasminogen activators, plasminogen activator inhibitor 1 and collagen type IV by immunofluorescence. Fixed cultures were blocked in phosphate-buffered saline/Tween for 5 min, then incubated with 5 μg/mL of primary antibody for 2 h at room temperature, washed twice in phosphate-buffered saline/Tween, then incubated with 20 μg/mL of secondary goat anti-mouse fluorescein isothiocyanate conjugate for 1 h. Unbound antibody was removed with three 5-min washes in phosphate-buffered saline/Tween.

Photography and analysis

Factor VIII-related antigen, *U. europaeus* agglutinin I, urokinase plasminogen activator, tissue plasminogen activator, plasminogen activator inhibitor 1 and collagen 4 expression by cells were assessed using a Leica DME compound microscope (Leica Microsystems GmbH Wetzlar, Germany) with an ultraviolet excitation attachment and a 460-nm filtration cube to detect emission by the fluorescein isothiocyanate-conjugated secondary antibodies. Dil-Ac-LDL uptake was assessed similarly using a 540-nm filtration cube. Fluorescent culture images were captured at 400× magnification in digital color using a Nikon 5 MP camera (Nikon, Melville, NY, USA) directly connected to the microscope. For each gingival sample and each characterization method, three random fields were used to count the number of positive individual cells vs. the total number of individual cells present, as represented by 4',6-diamidino-2-phenylindole staining, or as counted under phase-contrast microscopy. The percentages of positive cells in each field were then averaged to provide a per cent positive per gingival sample for each characterization method. The results are presented as means with standard deviations, or as ranges. Endothelial cell branching assays were also photographed at 400× magnification using a Nikon CoolPix 990 camera (Nikon), directly connected to a Leica DMIL inverted microscope (Leica Microsystems), with a phase-contrast condenser.

Data analysis

Experiments with each gingival endothelial cell line were repeated twice and an average value was assigned to each line. Mean values assigned to each of the six gingival endothelial cell lines were averaged and presented with the standard deviation.

Results

Gingival microvascular endothelial cells were isolated from six different nondiabetic participants where the average weight of the donor tissue samples was 144 ± 60 mg, or ≈ 140 mm^3. Each donor tissue sample from which gingival endothelial cell cultures could be established demonstrated at least mild histological inflammation. No gingival endothelial cell cultures could be established from firm, healthy, or fibrotic gingival biopsies.

Cultures of gingival endothelial cells, isolated as described herein, exhibited the classic 'cobblestone' morphology of endothelial cells as early as passage 1 (Fig. 1A). By the fourth passage, each successfully established gingival endothelial cell culture yielded $\approx 2.4 \times 10^6$ endothelial cells (range: $1.5–3.3 \times 10^6$ cells) in 75-cm^2 culture flasks. Dermal endothelial cell cultures also exhibited

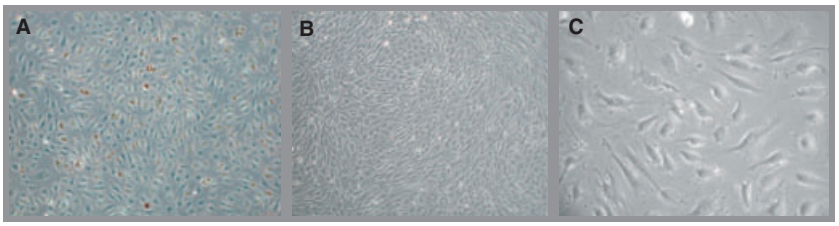

Fig. 1. Isolated gingival endothelial cells demonstrated a cobblestone culture phenotype. Phase contrast light micrograph (magnification ×400) of the culture morphology of gingival endothelial cells on plastic after passage 1. (A) Gingival cells were selected and plated as described in the Material and methods. A representative culture from one patient is shown. (B) Dermal endothelial cells, passage 9. (C) Human umbilical vein endothelial cells, passage 9.

a classic cobblestone appearance, similar in cell density to the gingival endothelial cells (Fig. 1B). A control human umbilical vein endothelial cell culture, by contrast, was loosely organized and less dense than the gingival cells (Fig. 1C). As determined by phase-contrast light microscopy, the gingival endothelial cells maintained their morphological phenotype from passage 2 to passage 9 (Fig. 2).

By passage 4, each of the isolated gingival endothelial cell cultures stained uniformly for Factor VIII-related antigen, with 92 ± 7% of the cells staining positively (Fig. 3A), which was similar to the dermal endothelial cells at passage 4 (Fig. 3B). Control wells of HDF were negative for Factor VIII-related antigen (Fig. 3C) as were gingival endothelial cells stained only with secondary antibody (data not shown). Human umbilical vein endothelial cells did not display Factor VIII binding antigen in our cultures (data not shown).

By passage 4, each of the isolated gingival endothelial cell cultures uniformly bound the lectin UEA-1 (93 ± 10%) (Fig. 4A), similarly to human umbilical vein endothelial cells (Fig. 4B) and dermal endothelial cells (Fig. 4C). Control dermal fibroblasts did not bind UEA-1 (Fig. 4D).

Dil-Ac-LDL uptake by live cultures after passage 4 demonstrated that 97 ± 4% of the cells in each gingival endothelial cell culture were positive for the uptake of the reagent (Fig. 5A), similarly to human umbilical vein endothelial cells (Fig. 5B) and dermal endothelial cells (Fig. 5C). HDF were quite limited in their ability to take up Dil-Ac-LDL (Fig. 5D).

When seeded upon Matrigel, the isolated gingival endothelial cell cultures began to form classical endothelial cell networks after 4 h of incubation (Fig. 6A), and networks were complete after 2 d, similarly to human umbilical vein endothelial cells (Fig. 6B) and dermal endothelial cells (Fig. 6C). No networks were formed by the control dermal fibroblasts up to 1 wk in culture (Fig. 6D).

Expression of plasminogen activator, plasminogen activator inhibitor 1 and collagen-4

Confluent cultures of the six gingival endothelial cells lines constitutively released variable basal levels of urokinase plasminogen activator, tissue plasminogen activator and plasminogen activator inhibitor 1 into the culture medium over 5 d. As expected, accumulation of plasminogen activator inhibitor 1 in the gingival endothelial cell culture medium (14 ± 10 nM) was orders of magnitude higher than that of urokinase plasminogen activator (60 ± 70 pM) or tissue plasminogen activator (40 ± 40 pM) measured in the wells (duplicate samples, experiments performed in triplicate). Similar expression was seen in dermal endothelial cells and human

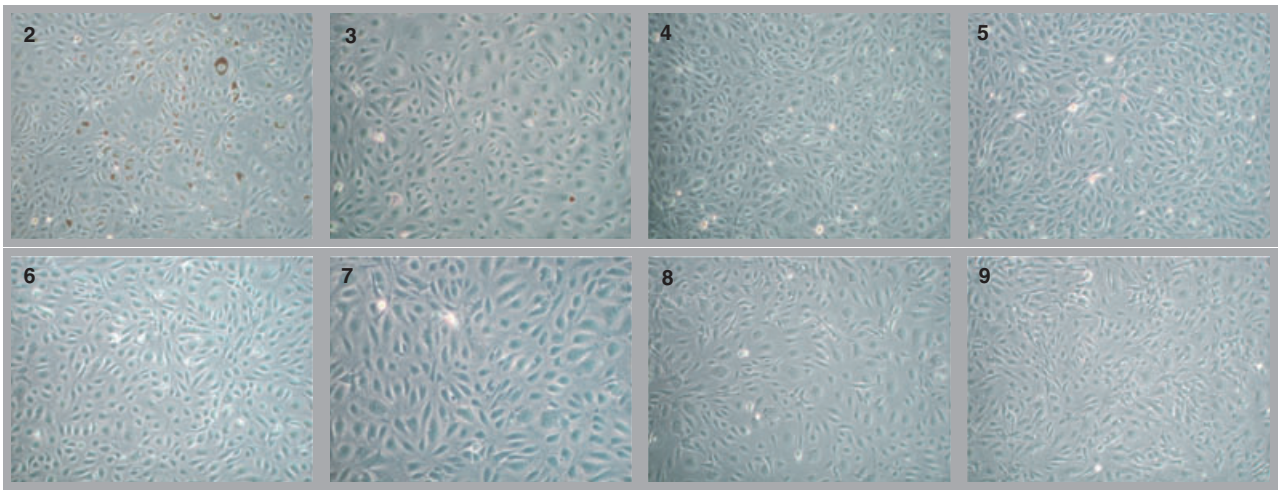

Fig. 2. Isolated gingival endothelial cells from passages 2–9. Phase-contrast light micrograph (magnification ×400) of the culture morphology on plastic of gingival endothelial cells between passages 2 and 9. Gingival cells were selected and plated as described in the Material and methods. A representative culture from one patient is shown.

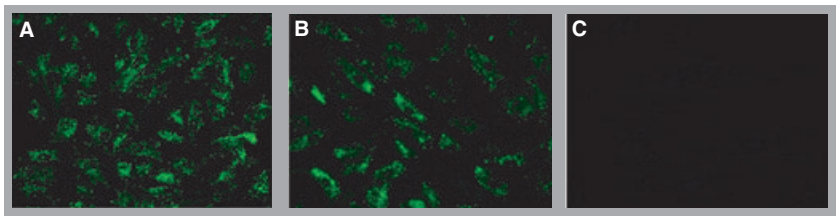

Fig. 3. Factor VIII-related antigen was localized in gingival endothelial cells (A) and in dermal endothelial cells (B). Dermal fibroblasts showed no immunoreactivity (C), similarly to control reactions in chambers of gingival endothelial cells incubated without primary antibody using only fluorescein isothiocyanate-conjugated secondary antibody (data not shown). Human umbilical vein endothelial cells (not shown) were also negative for Factor VIII-related antigen (data not shown). Images represent ×400 magnification.

umbilical vein endothelial cells (data not shown).

Cellular/extracellular matrix accumulation of urokinase plasminogen activator, tissue plasminogen activator, plasminogen activator inhibitor 1 and collagen type IV

Because much of the extracellular proteolytic activity is mediated through interactions with the cell surface, we also analyzed plasminogen

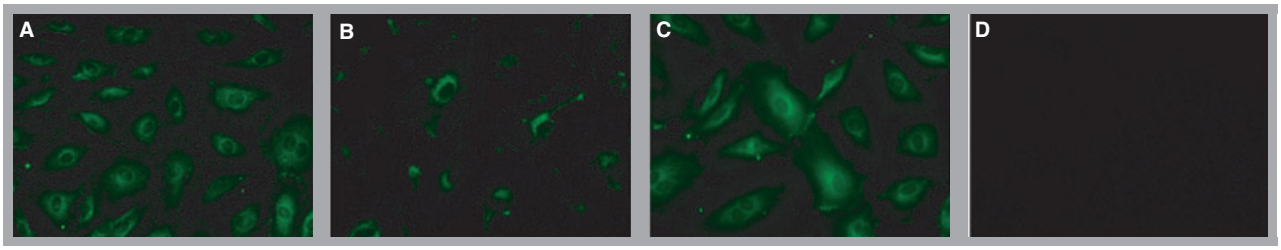

Fig. 4. UEA-1 ligand localization in gingival endothelial cells (A), human umbilical vein endothelial cells (B) and dermal endothelial cells (C). No reactivity was seen with dermal fibroblasts (D). A fluorescein isothiocyanate conjugate of UEA-1 was incubated with various cell types, as described in the Material and methods. Images represent ×400 magnification.

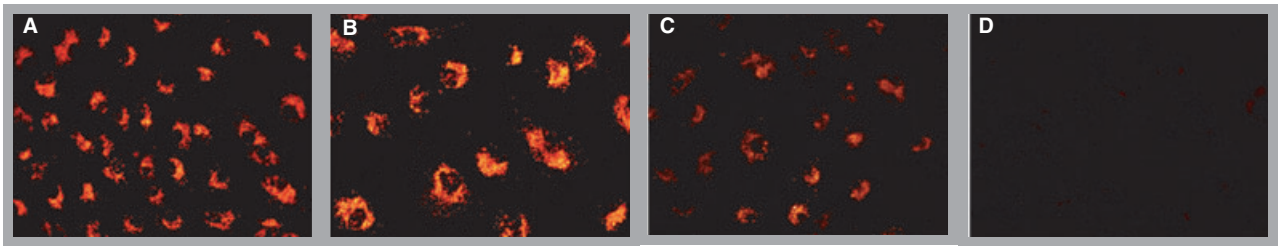

Fig. 5. Isolated gingival endothelial cells demonstrated the functional endothelial cell characteristic of acetylated low-density lipoprotein uptake. Gingival endothelial cells (A), human umbilical vein endothelial cells (B), dermal endothelial cells (C) and dermal fibroblasts (D) from the third passage were seeded in plastic culture wells and then assessed for the uptake of fluorescently labeled acetylated low-density lipoprotein over a 4-h period. Images represent ×400 magnification.

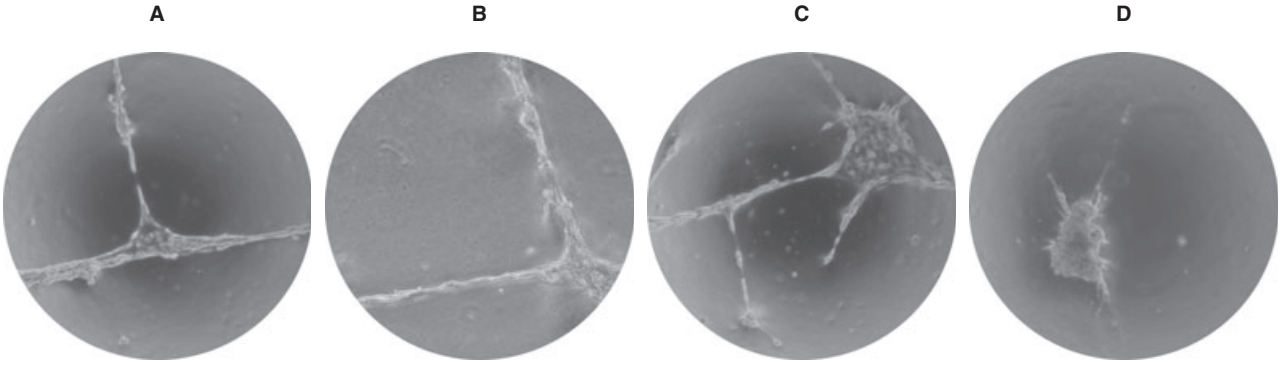

Fig. 6. Isolated gingival endothelial cells demonstrated the functional endothelial cell characteristic of network formation on growth factor-rich Matrigel substrate. Gingival endothelial cells (A), human umbilical vein endothelial cells (B), dermal endothelial cells (C) and dermal fibroblasts (D) from the third passage were seeded at a density of 10,000 cells/cm^2 on a thin layer of Matrigel and photographed under phase-contrast microscopy after 4 d of culture (magnification ×400).

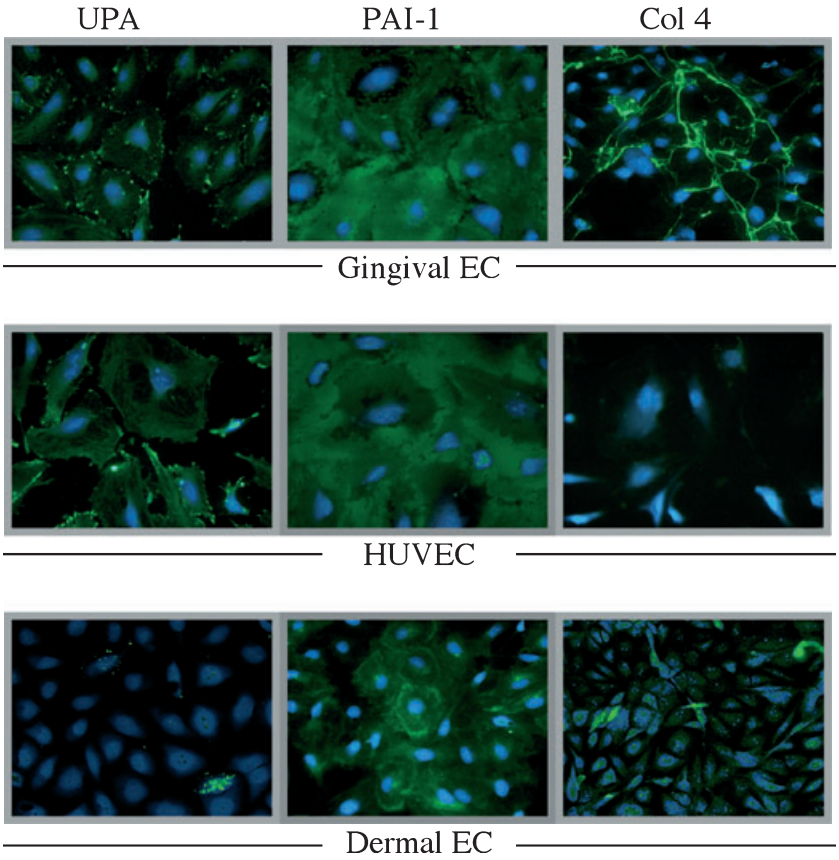

Fig. 7. Immunofluorescent localization of urokinase plasminogen activator (left column), plasminogen activator inhibitor 1 (middle column) and collagen type IV (right column) in gingival endothelial cells (top row), human umbilical vein endothelial cells (middle row), and dermal endothelial cells (bottom row). Cells were seeded on gelatin-coated slides and allowed to reach confluence during a 5-d incubation period before fixation in paraformaldehyde. Fluorescein isothiocyanate-labeled secondary antibodies identified urokinase plasminogen activator, plasminogen activator inhibitor 1 and collagen type IV on the slides, whereas the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (magnification ×400). Col 4, collagen type IV; Dermal EC, dermal endothelial cells; Gingival EC, gingival endothelial cells; HUVEC, human umbilical vein endothelial cells; PAI-1, plasminogen activator inhibitor 1; UPA, urokinase plasminogen activator.

activator and plasminogen activator inhibitor 1 levels in the cell monolayer and underlying substratum in the culture wells. All cell types expressed urokinase plasminogen activator strongly at focal adhesion points, and the detection levels appeared similar in gingival endothelial cells, dermal endothelial cells and human umbilical vein endothelial cells (Fig. 7). The pattern of plasminogen activator inhibitor 1 expression was also similar in all three cell types – strong and diffuse around the cell periphery and adjacent matrix. Only human umbilical vein endothelial cells displayed a significant tissue plasminogen activator signal, which was perinuclear (data not shown).

Primary gingival endothelial cells displayed a robust collagen type IV network pattern throughout the cultures; we detected a relative absence of collagen IV and no indication of a similar network pattern in our dermal endothelial cell and human umbilical vein endothelial cell cultures (Fig. 7).

Discussion

A detailed investigative approach to the gingival environment includes the use of primary gingival endothelial cells. With the advancement of tissue-engineering capabilities for oral structures (19), the availability of primary gingival endothelial cells seems requisite. We therefore needed to devise a relatively simple and reliable new method to isolate and enrich gingival endothelial cell populations.

For this, we used an adaptation of a published protocol describing isolation of heart microvascular endothelial cells with UEA-1-coated magnetic beads (13). The first change we made to the protocol was the use of anti-platelet-endothelial cell adhesion molecule 1 for selection, rather than UEA-1, in consideration of the importance that specificity has in minimizing competition from unwanted cells during initial culture. However, application of the protocol yielded insufficient numbers of endothelial cells from the gingival tissue samples to establish sustainable cultures. We then made three further significant modifications to the initial protocol, which increased the initial yield of endothelial cells from gingiva. First, because the 1-h digestion period left the less inflamed gingival tissue incompletely digested, we adopted an overnight digestion period. However, while the overnight treatment at 37°C was more effective in the digestion of the gingiva, it seemed detrimental to the cells recovered during the selection process and did not yield sufficient endothelial cell numbers for culture establishment. Therefore, a second protocol adjustment was made by lowering the level of collagenase during the overnight digestion period by 20-fold, coupled to an addition of 20 units of Dispase to the digestion cocktail. This modified cocktail appeared to be equally effective in gingival digestion and also appeared to be less damaging to the endothelial cells captured by the beads, hence improving their viability during initial plating and improving the probability of culture establishment from relatively small amounts of gingival tissue. Finally, the addition of 10% fetal bovine serum to the overnight digestion cocktail was felt to improve cell recovery further by lessening cell damage during the digestion period. Fine scalpel mincing of the gingival tissue before enzymatic digestion, and

constant gentle rotation during the digestion period, increased cell yield during the selection process. It is noteworthy that inflamed periodontal tissue samples digested better and produced greater cell yields. Donor samples of uninflamed or fibrous gingival tissue yielded no sustainable endothelial cell cultures.

A certain amount of endothelial cell cross-talk is evident in their ability to align and form networks. A minimum threshold cell density for endothelial cells to establish a sustainable culture is also apparently important and, for gingival endothelial cells, we estimate the need for ≈ 20 cells/cm^2 in two to three cell aggregates to be recovered from the initial bead selection process for successful gingival endothelial cell culture development. During subculture and seeding to subsequent passages, we also noted that the gingival endothelial cells ceased to proliferate if seeded below this critical density.

The anti-platelet-endothelial cell adhesion molecule 1 Dynal beads persisted in the cultures through the first few passages. However, the coating of the endothelial cells with the magnetic beads during the initial positive selection process, and the presence of some beads beyond the first passage, did not seem to affect the cell proliferation or function during long-term culture, making antibody-coated microbeads ideal for this procedure.

With these isolation and culture methods, the gingival endothelial cells proliferated to passage 17 before the cells stopped dividing. As described above, the cobblestone morphology of gingival endothelial cells was evident when selected cells reached initial confluence. It was noticed, however, that culturing these cells on plastic surfaces for longer than 2 mo could lead to a lengthening of the cell bodies and loss of the cobblestone colony morphology, which was recovered immediately when the cells were seeded on gelatin-coated plastic.

The endothelial cell cultures that we isolated did not demonstrate complete labeling for any of the markers chosen. This may be attributed to a normally small percentage of endothelial cells in culture that do not simultaneously display all three characteristics used in these analyses for quantification. For example, it was shown previously that lymphatic endothelial cells which express platelet-endothelial cell adhesion molecule 1 can demonstrate lower expression of at least one of the characteristics used here for endothelial identification (20). Alternatively, measurement of incomplete marker expression may also be attributed to nonendothelial cells, so it is recommended that cells isolated by this method should be characterized by investigators to ensure endothelial origin.

The relative excess of the inhibitor plasminogen activator inhibitor 1 to levels of protease urokinase plasminogen activator or tissue plasminogen activator released by endothelial cells in culture was expected based on prior experience with enzyme/inhibitor systems and from published data (21). Net proteolysis typically occurs in an environment of excess inhibitor through localization of protease by the cell. However, a relatively higher proportion of inhibitor to protease released extracellularly will affect the balance of the equation towards protease inhibition. Investigation of the effects of periodontal virulence factors on endothelial cells, especially those proteolytic in nature (22), may become more relevant using gingival endothelial cells.

The pattern of urokinase plasminogen activator distribution in the fixed endothelial cells was punctate, located at what appear to be primarily focal adhesion points, as expected (23), whereas the plasminogen activator inhibitor 1 distribution was diffuse with the appearance of extracellular accumulation, also as expected (24). Immunolocalization suggested that the gingival endothelial cells accumulated collagen type IV during the 5-d culture period. The pattern of collagen type IV deposition was strikingly different from that found for urokinase plasminogen activator or plasminogen activator inhibitor 1, and was similar to that previously shown to be deposited by certain epithelial cells (25). However, while these immunolocalization data compare plasminogen activator, plasminogen activator inhibitor-1 and collagen type IV expression among the endothelial cells seeded on gelatin, expression patterns of cellular and extracellular proteins in cultured cells can vary dramatically depending on the chosen substrate components upon which the cells were seeded (26).

Plasminogen activator and plasminogen activator inhibitor 1 expression by gingival endothelial cells presented here are only one factor in a complex balance of proteases to inhibitors, on the cell surface and in the extracellular matrix, determining the direction and specificity of protease activity and extracellular matrix remodeling. Although plasminogen activator inhibitor-2 was not measured in this characterization, it has been implicated clinically in periodontal tissue destruction (27) and could be modulated, at least in part, by endothelial cells of the gingiva (28). The interactions among proteases can be complex, with activation cascades involving different classes of proteases (such as the matrix metalloproteinases and the plasminogen activators with their respective inhibitors, tissue inhibitors of metalloproteinases (TIMPs) and PAIs (29). It is this net activity of cellular and extracellular proteases and inhibitors, sometimes at the leading edge of migrating cells (30), that corresponds with substrate remodeling and/or cell invasion. It is yet to be determined what role the gingival microvascular endothelial cells play in the net tissue destruction that accompanies microbial infection and inflammation of periodontal disease.

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