Human and Swine Dental Pulp Stem Cells Form a Vascularlike Network after Angiogenic Differentiation in Comparison with Endothelial Cells: A Quantitative Analysis

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Abstract

Introduction: The aim of this study was to quantify vascular network formation capacity after angiogenic induction of human and swine dental pulp stem cells (DPSCs) in comparison with endothelial cells. **Methods:** Primary human DPSCs or swine DPSCs were induced in endothelial growth medium for 7 days. The expression of the endothelial marker von Willebrand factor was determined by immunostaining. Induced DPSCs (iDPSCs) and noninduced DPSCs (niDPSCs) were seeded at different cell numbers onto Matrigel (BD Biosciences, San Jose, CA) for vascular network formation assays and analyzed after 4, 8, 12, and 18 hours in comparison with human microvascular endothelial cells (hMECs). Quantitative analysis of vascular tubule formation was performed using ImageJ software (National Institutes of Health, Bethesda, MD). The vascular network formation was also conducted by coculturing of niDPSCs and iDPSCs. **Results:** Von Willebrand factor was detected by immunofluorescence in both niDPSCs and iDPSCs (human and swine). Time-lapse microscopic observation showed that the vascular network was formed by iDPSCs but not niDPSCs. After 4 hours, iDPSCs showed vascular network formation, whereas niDPSCs started to aggregate and formed clusters. Human iDPSCs displayed a similar capacity to form vascular networks in Matrigel compared with hMECs based on quantitative analysis. Swine iDPSCs had a higher capacity compared with human iDPSCs or hMECs ($P < .05$) in forming the network structures including segments, nodes, and mesh. A coculture experiment showed that human niDPSCs colocalized on the angiogenic tubules and vascular networks that were formed by human iDPSCs. **Conclusions:** Our findings indicate that iDPSCs in combination with their noninduced counterparts may be used as a future clinical strategy for enhancing angiogenesis during the process of pulp-dentin regeneration. *(J Endod 2016;■:1–8)*

Key Words

Angiogenesis, coculture, dental pulp stem cells, Matrigel, tubule formation

Significance

This work was performed to test a potential clinical strategy for cell-based pulp-dentin regeneration by determining if we can induce DPSCs into endothelial-like cells. If yes, a combination of noninduced DPSCs and induced DPSCs may enhance the angiogenesis and regeneration of pulp dentin.

Angiogenesis plays a critical role in the success of engineered tissue regeneration. This is particularly important for cell-based therapy because the transplanted cells need blood supply for nutrients to survive in order to initiate tissue regeneration. In the case of cell-based pulp regeneration, angiogenesis in the root canal space faces a greater challenge because the source of any nutrient supply is restricted to the apical foramen. Because of this limitation, cell-based pulp regeneration was only considered possible if dealing with a tooth with a wide open apex. Even so, it was proposed that the pulp regeneration process may need to be incremental from the apical third toward the coronal third (1). Demonstrated from the proof-of-principle experiments, vascularized pulp tissue was regenerated using either a tooth slice model or a tooth fragment model in mice when the blood supply can be easily established during pulp regeneration (2, 3).

However, if we expect to use cell-based pulp regeneration on humans with most teeth having a small apical foramen, other regimens will be needed to achieve this goal. Several studies have reported that dental pulp stem cells (DPSCs) can express several angiogenic growth factors (4, 5), indicating their ability to induce angiogenesis. It has been shown that stem cells from human exfoliated deciduous teeth have the potential to become endothelial-like cells evidenced by the expression of endothelial cell (EC) markers *in vivo* (6). Limited evidence has also indicated that DPSCs have the potential to differentiate into endothelial-like cells (7–10). However, no effort has been made to use this premise as a strategy in the context of enhancing pulp regeneration. In this study, we took the first step by using an *in vitro* system to test whether angiogenically induced DPSCs form a vascular network in comparison with that of ECs. Coculturing of noninduced DPSCs (niDPSCs) and induced DPSCs (iDPSCs) was also conducted to assess the spatial relationship between niDPSCs and iDPSCs during vascular network formation.

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Materials and Methods

Cell Culture

Freshly extracted human and swine teeth were stored in serum-free alpha minimum essential medium (HyClone, Logan, UT) and transported to the laboratory for processing using a previous published protocol (3). Human teeth were from 15- to 25-year-old healthy donors in the oral surgery clinics at Boston University, Boston, MA, or the University of Tennessee Health Science Center (UTHSC), Memphis, TN. The patient sample collection in this study conformed to exempt protocols approved by the Institutional Review Board of Boston University (#H-28882) and UTHSC (12-01937-XM). Swine teeth were collected from Sinclair miniature swine (12- to 17-month-old females; Sinclair Bio-Resources, Auxvasse, MO [http://www.sinclairbioresources.com/]). Animal procedures followed a protocol (#2092) approved by the Institutional Animal Care and Use Committee at UTHSC. Pulp tissue was removed from teeth, minced, and digested in a solution of 3 mg/mL collagenase type I and 4 mg/mL dispase (Sigma-Aldrich, St Louis, MO) for 30 to 60 minutes at 37°C. Cells were seeded into 6-well plates and cultured in a standard medium with alpha minimum essential medium supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA), 2 mmol/L L-glutamine, 100 mmol/L L-ascorbic acid 2-phosphate, and 1% antibiotic antimycotic (Life Technologies, Grand Island, NY) and maintained in 5% CO_2 at 37°C. The isolated heterogeneous population of DPSCs was passaged at a 1:3 ratio when they reached 80% confluence and expanded to passage 3 for experimentation. Human microvascular endothelial cells (hMECs) (passage 9, obtained from Dr Yi Lu, UTHSC [11]) were cultured in endothelial growth medium (EGM-2; Lonza, Walkersville, MD) until 80% confluency for experimentation.

Endothelial Induction of DPSCs

Human or swine DPSCs at passage 3 and 70% confluency were cultured under standard (noninduced) or angiogenic (induced) conditions. For the angiogenic condition, cells were cultured with EGM-2 for 7 days.

Immunofluorescence

Cells were cultivated in wells of 24-well plates coated with a thin layer of Matrigel (3 mg/mL, Basement Membrane Matrix; BD Bioscience, San Jose, CA), fixed with methanol at −20°C for 10 minutes, and rinsed with phosphate-buffered saline. Cultures were incubated with a primary antibody against the endothelial cell marker von Willebrand factor (vWF) (rabbit antihuman, 1:100 dilution; Sigma-Aldrich, cat# F3520) overnight, washed with phosphate-buffered saline, and incubated with Alexa Fluor–conjugated goat antirabbit immunoglobulin G (cat#ab150077; Abcam, Cambridge, MA) for 30 minutes. Counterstaining of the cells with 4′,6-diamidino-2-phenylindole was performed, and the cells were observed under fluorescence microscopy. The quantification of the fluorescence intensities was performed using the fluorescence images and ImageJ software (National Institutes of Health, Bethesda, MD). Three images taken from 3 randomly selected areas in the stained cell cultures per group were measured. In each image, the outline of each cell was drawn, and the area, mean fluorescence, and background readings were measured (12, 13). The total cellular fluorescence was calculated using the following formula: total cellular fluorescence = integrated density − (area of selected cells × mean fluorescence of background readings).

In Vitro Angiogenesis Tubule Formation Assay

Ninety-six–well plates were coated with 50 μL/well chilled Matrigel solution (10 mg/mL) without air bubbles and incubated for 1 hour at 37°C to solidify. niDPSCs, iDPSCs, or hMECs were trypsinized and seeded onto the solidified Matrigel in the wells at different cell numbers (1×10^4, 1.5×10^4, 2×10^4, or 3×10^4 cells/well or 353, 530, 706, 1060 cells/mm^2, respectively) in 150 μL EGM-2 medium and incubated at 37°C. Endothelial tubulelike vascular network formation was observed at 4, 8, 12, and 18 hours of incubation under an inverted microscope.

The images were captured at ×4 in the phase contrast mode, and the vascular network was analyzed using ImageJ software according to a method reported by Chevalier et al (14). Briefly, the vascular networks were segmented and skeletonized. The trees were then analyzed by the detection of nodes (the branching points), segments (the number of master segments), mesh (the closed loops), and the total length of tubules of the cellular meshed network organization (15).

Coculture of Human niDPSCs and Human iDPSCs

Human niDPSCs at passage 1 were seeded into the wells of 12-well plates and transduced with pLenti CMV green fluorescent protein (GFP) vectors (7×10^8 IU/mL; UCLA Vectorcore, Los Angeles, CA) at a multiplicity of infection of ∼30 with the presence of polybrene (4 μg/mL). Human niDPSCs at passage 3 were labeled with Vybrant Dil (V2288; Invitrogen, Carlsbad, CA) (denoted as human niDPSCs-VD). Human niDPSCs-GFP expanded to passage 3 were cultured with EGM-2 for 7 days for angiogenic induction (human iDPSCs-GFP). A 96-well plate was coated with 50 μL/well Matrigel. Human iDPSCs-GFP (1.5×10^4) in 100 μL EGM-2 medium were seeded onto Matrigel in each well, immediately followed by seeding human niDPSCs-VD in 50 μL/well standard medium into the same well. Various cell ratios of human iDPSCs-GFP:human niDPSCs-VD were used (ie, 2:1, 5:1, 10:1, and 20:1); human iDPSCs-GFP stayed at 1.5×10^4 cells/well. The well was then mixed by swirling. Network formation and localization of cells were observed under a fluorescence microscope at 4 hours after cell seeding.

Data Analysis

One-way analysis of variance was used to compare a single factor among 3 groups for the quantitative analysis of cell fluorescence intensity and angiogenesis network formation. When the main effect was found to be statistically significant, post hoc comparisons were examined using the Tukey honest significant difference test. Data are reported as mean ± standard deviation, and values are considered significant when $P < .05$. All analyses were performed using SPSS software (SPSS Inc, Chicago, IL).

Results

Endothelial Marker Expression of iDPSCs

After 7 days of angiogenic induction, iDPSCs exhibited slight morphologic changes. The cell body tended to be less spread out, resembling hMECs. Human iDPSCs exhibited elongated cell extensions between cells, which was also noted in hMECs. In general, swine niDPSCs showed less spindle-shaped morphology but a more triangular or trapezoid shape compared with human counterparts. After induction, they appeared more ovoid and round (Fig. 1*A*). Immunofluorescence staining of the EC marker vWF revealed that both niDPSCs and iDPSCs expressed the marker (Fig. 1*B*). The quantification of the fluorescence intensities showed that hMECs expressed significantly higher levels of vWF than human niDPSCs but not the other cell groups. No difference was detected between the noninduced and induced groups (Fig. 1*C*).