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Mice dental pulp and periodontal ligament endothelial cells exhibit different proangiogenic properties

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ABSTRACT

Dental pulp is a highly vascularized tissue with a high regenerative capacity. This is attributed to its unique blood supply and the presence of progenitor or postnatal dental pulp stem cells. Here we aimed to isolate and compare the angiogenic properties of endothelial cells (EC) prepared from mouse dental pulp and periodontal ligament (PDL). EC were isolated from 4-week-old wild type immorto mice. Mice were sacrificed and after mandible isolation, the molar and incisor teeth and the PDL from molar teeth were dissected. EC were prepared by collagenase digestion of tissues and affinity purification using magnetic beads coated with platelet/endothelial cell adhesion molecule-1 (PECAM-1/CD31) antibody. EC prepared from incisor and molar pulps and PDL were examined for expression of appropriate markers by fluorescence-activated cell sorting (FACS) analysis. The proliferation, migration, and capillary morphogenesis of EC were evaluated. Ex vivo sprouting angiogenesis from various tissues was also compared. Data were analyzed at the level of significance of $P < 0.05$. Pulp EC prepared from incisors proliferated and migrated significantly faster than molar and PDL EC ($P < 0.05$). In addition, molar and PDL EC formed a more extensive capillary network when plated on Matrigel. This is consistent with the lower proliferative and migratory characteristics of these cells compared with incisor EC ($P < 0.05$). However, PDL tissue showed significantly more sprouting area than molar and incisor pulp tissues ($P < 0.05$). Thus, pulp EC from molar and incisor and PDL EC present different proangiogenic properties. Collectively our results suggest that EC from different tooth tissue have unique characteristics related to their target tissue and function.

1. Introduction

Endothelial cells (EC) line the surface of whole vascular network starting from heart to the smallest capillaries, which control the passage of white blood cells and different substances into or out of the blood vessels. Besides this primary role as a permeable barrier for blood vessels, EC perform critical functions including regulation of hemostasis, the coagulation cascade, vascular tone, and immunity (Aird, 2007; Asahara et al., 1997; Saghiri et al., 2015a). Moreover, EC play a critical role in tissue regeneration through the process of angiogenesis. Angiogenesis is a multistep process involving the growth of new blood vessels from pre-existing capillaries, and is tightly regulated by the production of various angiogenic regulatory factors (Folkman, 1984; Risau, 1997). Some of these factors are released by the surrounding tissues in response to ischemia, which activate nearby EC and initiate the process. This process includes degradation of extracellular matrix, proliferation and migration of EC, and ultimately establishment of tubular structures (Faller, 1999).

Human dental pulp is a highly vascularized tissue that has a high regenerative capacity due to its unique blood supply and the presence of progenitor or postnatal dental pulp stem cells (DPSC) (Gronthos et al., 2000; Nakashima et al., 2009; Saghiri et al., 2015b). DPSC are recruited and differentiate into odontoblast-like cells to produce reparative dentin (Saghiri et al., 2015c). Vascularization plays a key role in determining whether injured pulp tissue survives or undergoes necrosis (Mantellini et al., 2006). In addition to participation in sprouting angiogenesis, it was demonstrated that EC may have a synergistic effect on DPSC. The co-culture of DPSC and EC enhanced osteogenic and odontogenic properties of DPSC on one hand, and promoted the establishment and longevity of pre-existing blood vessel-like structures formed by EC on the other (Dissanayaka et al., 2012). Thus, the dental pulp EC and vasculature play a pivotal role in survival and regeneration

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of dental pulp after injuries.

The periodontium is defined as the tooth supporting structures including root cementum, periodontal ligament (PDL), alveolar bone, and the dentogingival junction. PDL is the soft and specialized connective tissue in periodontium, which contains well-defined and oriented collagen fiber bundles connecting the root cementum to the alveolar bone. PDL consists of several cell types including fibroblasts, cementoblasts, odontoclasts, osteoblasts, osteoclasts, undifferentiated mesenchymal cells, epithelial cell rests of Malassez, macrophages, and monocytes (Nanci and Bosshardt, 2006). In addition to these components, PDL has a complex and well-distributed vascular network derived from the alveolar bone and gingiva, which is oriented in two layers including the outer or peripheral and the inner layers. The outer layer consists of larger vessels, which play a great role in shock absorbing system, while the inner layer contains the capillary vessels that supply PDL structures with oxygen and nutrients (Masset et al., 2006). Besides the crucial functions of vascular networks, the EC of PDL vessels were shown to play an important role in proliferation of PDL and gingival fibroblasts (Jin and Yuan-zheng, 2013). It was also demonstrated that EC from PDL have more permeability compared to EC from other tissues (Maruyama and Sato, 2016). Thus, PDL EC cooperate with other cells in maintaining the function and regeneration of PDL.

Based on the importance of EC in dental pulp and PDL regeneration, and the information gap regarding the properties of these cells, the present study aimed to evaluate and compare the characteristics of EC prepared from mice PDL and the dental pulp from incisors and molar teeth. We determined whether EC from the different tooth tissues had different properties, which may contribute to their angiogenic potential during regenerative and reparative procedures. These studies will advance our knowledge regarding the regulatory tissue specific function of these EC, and could aid in the development of new and effective therapies for regenerative and reparative dentistry.

2. Material and methods

2.1. Experimental animals

All the experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology statement for the use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and use Committee of the University of Wisconsin School of Medicine and Public health. Immorto mice expressing a temperature-sensitive simian virus (SV) 40 large T antigen were obtained from Charles Rivers Laboratories (Wilmington, MA) and backcrossed to a C57BL/6J background and maintained as previously described (Lawler et al., 1998; Su et al., 2003). The isolated DNA from tail biopsies was used for screening of various transgenes as previously described (Scheef et al., 2009; Su et al., 2003).

2.2. Isolation and culture of EC

EC were isolated form one litter (6 or 7 pups) of 4-week-old mice. Briefly, first molar and incisor teeth were harvested. The molar and incisor tooth were broken at the cement enamel junction (CEJ), and the pulp tissue were collected under a dissecting microscope. Periodontal ligament (PDL) tissues isolated from mandibular first molars of same mice. The PDL tissues were separated from the root surface using a scalpel and were minced into the smallest size possible. All tissues were cut up using a razor blade and then digested in 5 mL of Collagenase type I (Worthington, Lakewood, NJ; 1 mg/mL in serum-free Dulbecco's modified Eagle's medium; DMEM) and incubated at 37 °C for 40 min. **(Supplementary Movie)**. The digested tissues were washed with DMEM containing 10% fetal bovine serum (FBS), and centrifuged. The pellet was re-suspended in 10 mL of DMEM with 10% FBS and passed through a nylon mesh with a pore size of 70 μm. The cells were pelleted and re-suspended in 1 mL of DMEM with 10% FBS and mixed with magnetic beads coated with PECAM-1 antibody prepared as previously described (Su et al., 2003; Zerfaoui et al., 2008). The mixture allowed to rock at 4 °C for 1 h. Following incubation, the beads were collected using a magnet and washed six times with 1 mL of DMEM with 10% FBS. The beads were then re-suspended in 0.5 mL of EC growth medium as previously described (Su et al., 2003). The EC growth medium contained DMEM with 10% FBS, 2 mM L-glutamine, 2 mM sodium pyruvate, 20 mM HEPES, 1% non-essential amino acids, 100 μg/ml streptomycin, 100 U/ml penicillin, freshly added heparin at 55 U/ml (Sigma, St. Louis, MO), endothelial growth supplement 100 μg/ml (Sigma), and murine recombinant interferon-γ (R&D, Minneapolis, MN) at 44 units/ml.

Cells were maintained at 33 °C with 5% CO_2. Cells were progressively passed to larger plates, maintained, and propagated in 1% gelatin-coated 60 mm dishes. Three different isolations of EC were used in these studies and all cells were used prior to passage 15.

2.3. Fluorescence activated cell sorting (FACS) analysis

EC form 60-mm culture plates were rinsed with PBS containing 0.04% EDTA and incubated with 1.5 mL of Cell Dissociation Solution (Sigma, St. Louis, MO). Cells were then washed, collected from plates with DMEM containing 10% FBS, centrifuged, and blocked in 0.5 mL of Tris-buffered saline (TBS; 25 mmol/L Tris- HCl, 150 mmol/L NaCl, pH 7.6) with 1% goat serum for 20 min on ice. Cells were then pelleted and incubated in 0.5 mL TBS with 1% BSA containing a specific primary antibody on ice for 30 min. The following antibodies were used: anti-VE-cadherin (Enzo Life Sciences; Farmingdale, NY), anti-PECAM-1 (BD Biosciences; San Jose, CA), and B4-lectin (Vector Laboratories; Burlingame, CA) at dilutions recommended by the supplier. Cells were then rinsed twice with TBS containing 1% BSA and incubated with appropriate FITC-conjugated secondary antibody (Pierce, Rockford, IL) prepared in TBS containing 1% BSA for 30 min on ice. Following incubation, cells were washed twice with TBS containing 1% BSA, re-suspended in 0.5 mL of TBS with 1% BSA and analyzed by a FACScan caliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ). These experiments were repeated twice using two different isolations of EC with similar results.

2.4. Cell proliferation assay

Cell proliferation was assessed by counting the number of cells over a two weeks period. Cells (1×10^4) were plated in multiple sets of gelatin-coated 60-mm tissue culture plates, fed every other day for the duration of experiment. The number of cells was determined by counting every other day, on days not fed, in triplicates.

2.5. Scratch wound assay

Cells (1×10^6) were plated in 60-mm tissue culture dishes and allowed to reach confluence (1–2 days). Plates were wounded using a 1-mL micropipette tip, washed with growth medium twice to remove detached cells, and fed with growth medium containing 1 μmol/L 5-fluorouracil (Sigma) to block cell proliferation. Wound closure was monitored by phase microscopy at different time points (0, 24, 48 h) and images were captured in digital format. The migrated distance as percentage of total distance was determined for quantitative assessment of data as described previously (DiMaio and Sheibani, 2008).

2.6. Transwell migration assay

Cell migration was also determined using a transwell migration assay. Costar transwell inserts (8-μm pore size, 6.5-mm membrane, Lowell, MA) were coated with serum free DMEM containing fibronectin (2 μg/mL) on the bottom side at 4 °C overnight. After washing with PBS, inserts were blocked in PBS containing 1% BSA for 1 h at room