Local Injection of Pulp Cells Enhances Wound Healing during the Initial Proliferative Phase through the Stimulation of Host Angiogenesis

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Abstract

Introduction: The aim of this study was to examine the effect of pulp cell injection on host angiogenesis during wound healing. **Methods:** Pulp cells were isolated from extracted premolars by the outgrowth method. Fluorescently labeled pulp cells or phosphate-buffered saline were locally injected into a mouse wound healing model. Wound healing was evaluated using photographs, histology, and real-time reverse-transcription polymerase chain reaction. Injected cells were traced. Angiogenesis was measured by performing immunohistochemical staining of CD31, a marker of vascular endothelial cells. The level of secreted vascular endothelial growth factor in the pulp cell conditioned medium (CM) was compared with the CM of fibroblasts and keratinocytes. The paracrine effect of pulp CM on angiogenesis was evaluated by tubular network formation using endothelial cells. **Results:** The local injection of pulp cells enhanced wound closure during the initial stage when compared to the injection of phosphate-buffered saline. The amount of extracellular matrix production and the expression of CD31+ cells were also increased in response to pulp cell injection when compared with the injection of phosphate-buffered saline. The fluorescently labeled pulp cells were engrafted into the hair follicles of the adjacent normal dermis but not into the wound site per se. A significantly higher level of vascular endothelial growth factor was secreted into the CM of pulp cells when compared with dermal fibroblast and keratinocytes. Tubular network formation of endothelial cells and the proliferation of dermal fibroblasts were significantly enhanced by the application of pulp cell CM when compared with control media. **Conclusions:** Our results show that local injection of pulp cells is effective in enhancing wound healing during the initial proliferative phase, especially through paracrine mechanisms regulating host angiogenesis and proliferation. *(J Endod 2013;39:788–794)*

Key Words

Paracrine effect, pulp stromal cell, wound healing

With the expansion of regenerative medicine and tissue engineering therapies, the regeneration of soft and hard tissue has been of interest to many clinicians. Stem cells with rapid self-proliferation rates and pluripotent ability have been isolated from dental organs, including the dental pulp, periodontal ligaments, pulp from exfoliated deciduous tooth (SHED), and apical papilla, and reportedly regenerate periodontal hard tissues *in vitro* and *in vivo* (1). The potent application of dental stem cells has also been suggested for the regeneration of soft tissue, such as the dentin-pulp complex (2–4), the promotion of healing of cutaneous wounds (5, 6), and skin rejuvenation (7).

Wound healing is a dynamic response in living bodies composed of migration, the proliferation of various regulators and cells, vascular formation, and the remodeling process. Angiogenesis is a necessary process during the proliferation phase of wound healing, which provides fibroblasts with a sufficient nutrient supply for the production of granulation matrix (8). Without proper angiogenesis, the prognosis of the wound becomes poor, resulting in nonhealing chronic wounds, such as the diabetic foot (9). Along with angiogenesis, hematopoietic stem cells and multipotent mesenchymal stem cells (MSCs) are transported from the marrow to regenerate damaged tissue (10). In addition to the host stem cells, the local application of various MSCs has shown therapeutic potential for these cells in wound healing through differentiation into fibroblasts or keratinocytes, further promoting angiogenesis and host tissue remodeling via the secretion of growth factors (11). Several clinical trials also support the positive effect of MSCs in nonhealing chronic wounds (9, 12).

Dental pulp is a well-vascularized connective tissue that has the potential to heal in response to various injuries (13, 14). Similar to other MSCs, pulp cells secrete high levels of angiogenic factors, such as vascular endothelial growth factor (VEGF) (15), and its injection *in vivo* increases blood flow through functional revascularization (16, 17). Given that pulp cells express proangiogenic MSC-like properties, we hypothesized that the application of pulp cells may enhance soft-tissue regeneration through the stimulation of angiogenesis. Therefore, the purpose of this study was to evaluate the stimulatory

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effect of pulp cells on host angiogenesis during soft-tissue regeneration by using a well-established *in vivo* model of wound healing.

Materials and Methods

Pulp Cell Isolation and Collection of Conditioned Media

Premolars extracted for orthodontic purposes were collected from 4 patients at the Department of Orthodontics, Gangnam Severance Dental Hospital, Yonsei University, Seoul, Korea, under approved guidelines set by the institutional review board. Pulp cells were obtained from isolated tissues using an outgrowth method in αMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS), 100 U penicillin, and 100 mg streptomycin (18). Cells between passages 3 and 7 were used in this study. Previously, it was shown that the outgrown pulp cells were CD90 and 105 positive; self-replicative; and capable of differentiating into adipogenic, osteogenic, and chondrogenic lineages as seen with other MSCs (18). To collect the conditioned media (CM), human dermal fibroblasts (HDFs [Invitrogen]) and immortalized human oral keratinocyte (IHOK [provided by professor J Kim, Yonsei University]) were cultured in the same conditions as the pulp cells in αMEM. CM was collected during cell passage using 0.22-μm syringe filters.

Lentiviral Transduction

Lentiviral vector-expressing fluorescent green fluorescent protein was purchased from Seoulinbioscience (Seongnam, Korea). Pulp cells were plated in 60-mm culture dishes in αMEM with 10$\times$ concentrated virus (10 μL/dish) for 24 hours (19).

In Vivo Wound Healing Model and Cell Application

Seven-week-old ICR male mice (Orient Bio, Seongnam, Korea) were used. The study was approved by the Institutional Animal Care and Use Committee at Yonsei University. After intraperitoneal anesthesia using Zoletil 50 (Virbac, Carros, France) at a concentration of 30 mg/kg and Rompun (Bayer, Hanam, Korea) at a concentration of 10 mg/kg, dorsal hair was removed, and an 8-mm full-thickness defect was created by a skin biopsy punch (Kai Ind, Tokyo, Japan). Pulp cells (2.0×10^6) suspended in 300 μL phosphate-buffered saline (PBS) (pulp cell injection group, $n = 16$) or an equivalent amount of PBS alone (sham control group, $n = 16$) were injected immediately after wound creation at 8 points along with the boundary of the wound using a 26-G syringe (20). Tegaderm (3M Health Care, St Paul, MN) was applied to prevent dehydration surrounding the wound site.

Wound Healing Analysis and Pulp Cell Detection

Digital photographs of the wound site were taken on days 0, 3, 6, 9, 12, and 14. Wound size was measured using ImageJ software (National Institutes of Health, Bethesda, MD). The wound area was calculated as the percent area of the original wound (6, 20).

Histologic Analysis and Tracing of Injected Pulp Cells

Experimental animals were sacrificed on days 7 and 14 ($n = 4$ for each group) after wound formation to make frozen sections of the surrounding tissue in 4-μm intervals. Hematoxylin-eosin (H&E) staining was performed for optical microscopy. In order to measure cellularity of the granulation tissue, the number of cells per 300 μm^2 within the granulation tissue near the wound margin directly under the epithelium was counted using computer-assisted planimetry (Metamorph Ltd, Limerick, Ireland). Tracing of the injected pulp cells was performed using confocal microscopy (LSM710; Carl Zeiss Inc, Jena, Germany) after counterstaining using nuclear dye 4′, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc, Burlingame, CA).

Real-time Reverse-transcription Polymerase Chain Reaction Analysis

Total RNA was isolated from the dorsal skin specimens of the wound site using Trizol reagent (Invitrogen) and 1 μg total RNA was converted to complementary DNA using a TaKaRa RNA PCR Kit Ver.2.1 (TaKaRa Bio Inc, Shiga, Japan) and the following reaction conditions set by the manufacturer: 45°C for 45 minutes and 95°C for 5 minutes. Real-time polymerase chain reaction (PCR) amplifications were performed using Light Cycler 480 SYBR Green (Roche Diagnostics GmbH, Mannheim, Germany) in a LC480II (Roche Diagnostics GmbH) following the manufacturer's protocol. The annealing temperature was 52°C for *MMP1* and 60°C for the other genes. The data were analyzed using LightCycler 480 software (Roche Diagnostics GmbH). Messenger RNA (mRNA) expression of each gene was normalized to that of the housekeeping gene mouse beta-actin. The primers used for real-time reverse-transcription PCR are as follows: mCollagen 1, forward: AGCGGAG AGTACTGGATCG, reverse: GCTCTTTTCCTTGGGGTTC; mCollagen 3, forward: AGGCTGAAGGAAACAGC AAA, reverse: TAGTCTCATTG CCTTGCGTG; mTGF-β1, forward: CACCGGAGAGCCCTGG ATA, reverse: TGCCGCACACAGCAGTTC; mVEGF, forward: ACTGGACCCTGGCTTTA CTG, reverse: TCTGCTCTCCTTCTGTCGTG; and mMMP1, forward: CCTTCCTTTGCTGTT GCTTC, reverse: CACCTGGGCTTCTTCATAGC. Relative messenger RNA abundances of target genes in the pulp cell injection group ($n = 4$) were compared with that of the sham control group ($n = 4$).

Immunofluorescence of CD31

After application of the protein block solution (DAKO, Glostrup, Denmark), the primary antibody diluted to 1:100 (purified rat antimouse CD31; BD Biosciences, San Jose, CA) was applied to react overnight at 4°C. The secondary antibody diluted to 1:400 (Alexa Fluor 555 goat antirat IgG, Invitrogen) was applied for 30 minutes. DAPI mounting solution was used for counterstaining. The optical density of CD31 was measured as pixels against the overall wound surface using computer-assisted planimetry (Metamorph, Metamorph Ltd).

VEGF Immunoassay

Soluble VEGF was measured using a quantitative sandwich enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Tubular Network Formation Assay

Because growth media for human umbilical vein endothelial cells (HUVECs) is conventionally endothelial growth medium-2 (EGM-2) with 2% FBS, we also maintained pulp cells in EGM-2 with 2% FBS in addition to αMEM with 2% FBS and collected the resulting culture soup for the CM (11). For tubular network formation, a 24-well plate was coated with 250 μL chilled BD Matrigel basement membrance (BD Bioscience, Billerica, MA) (9.1 mg/mL) per well and incubated at 37°C for 60 minutes; 1.2×10^5 HUVECs were suspended in 0.4 mL of control media (αMEM and EGM-2) or CM of pulp cells in αMEM and EGM-2. The cells were seeded and cultured at 37°C, 5% CO_2 atmosphere for 24 hours.

Cell Proliferation Analysis

HDFs were seeded in a 96-well plate at a concentration of 4×10^6 cells per well in triplicate with 50 μL αMEM with or without