Evaluation of the Delivery of Mesenchymal Stem Cells into the Root Canal Space of Necrotic Immature Teeth after Clinical Regenerative Endodontic Procedure

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Abstract

Introduction: Immature teeth with open apices treated with conventional nonsurgical root canal treatment often have a poor prognosis as a result of the increased risk of fracture and susceptibility to recontamination. Regenerative endodontics represents a new treatment modality that focuses on reestablishment of pulp vitality and continued root development. This clinical procedure relies on the intracanal delivery of a blood clot (scaffold), growth factors (possibly from platelets and dentin), and stem cells. However, to date, the clinical presence of stem cells in the canal space after this procedure has not been demonstrated. The purpose of this clinical study was to evaluate whether regenerative endodontic procedures are able to deliver stem cells into the canal space of immature teeth in young patients and to identify the possible tissue origin for these cells. Methods: After informed consent, the first appointment consisted of NaOCl irrigation and treatment with a triple antibiotic paste. One month later, the root canal space was irrigated with sterile saline, and bleeding was evoked with collection of samples on paper points. Real-time reverse-transcription polymerase chain reaction and immunocytochemistry were conducted to compare the gene transcripts and proteins found in the root canal sample with levels found in the systemic circulation. Results: Molecular analyses of blood collected from the canal system indicated the significant accumulation of undifferentiated stem cells into the canal space where these cells might contribute to the regeneration of pulpal tissues seen after antibiotic paste therapy of the immature tooth with pulpal necrosis. (J Endod 2011;37:133–138)

Key Words

Confocal, dental pulp, genotype, immature necrotic teeth, mesenchymal stem cells, regenerative endodontics, revascularization, RT-PCR, SCAP, stem cell therapy.

Endodontic therapy in permanent teeth diagnosed with pulpal necrosis and immature root development is fraught with challenges. The primary objective of endodontic therapy in infected root canal systems is to disinfect the root canal system through chemical and mechanical means (1). However, in teeth with immature root development, the removal of microorganisms by mechanical means is limited as a result of the thin, fragile dentinal walls of the roots. Disinfection of the root canal system in these cases often relies on irrigation and intracanal medicaments. After completion of traditional endodontic therapy, these teeth often have a poor crown-to-root ratio and are susceptible to fracture (2, 3). Recently, an alternative, biologically based, regenerative approach has been advocated on the basis of prior revascularization studies from the trauma literature (4–7). The publication of several case series has led to growing recognition of the potential for successful outcomes by using regenerative procedures in treating the necrotic immature permanent tooth (8–11).

Multiple studies showed continued root development can be accomplished after disinfection of the root canal system, evoked bleeding inside the root canal, and adequate coronal seal (8, 10, 12–17). The use of these treatment protocols can result in radiographic and clinical evidence of healing and subsequent root development that has been attributed to a regeneration of tissue. The exact mechanism and source of the cells responsible for the radiographic evidence of continued root development remain unknown. However, the identification of an enriched population of mesenchymal stem cells within the apical papillae (SCAP) of immature teeth has led to the suggestion that these cells might contribute to the regenerative response that follows these clinical procedures (18). Although regenerative protocols include a step that induces bleeding within the canal space after the manipulation of periapical tissues, it is not known whether this step triggers the release of stem cells into the root canal space. This issue represents an important gap in knowledge because tissue engineering involves the controlled delivery of stem/progenitor cells as well as a scaffold and growth factors (18), so knowledge regarding the potential source of stem cells is critical to the further development of pulpal regenerative...
procedures. Some have even questioned the possible role of stem cells in these regenerative responses, and so the possible presence of stem cells within the root canal space after a regenerative procedure needs to be evaluated. Accordingly, in the present study we evaluated the delivery of mesenchymal stem cells into root canal systems of immature necrotic permanent teeth in human subjects.

### Materials and Methods

#### Patient Recruitment

The Institutional Review Board of the University of Texas Health Science Center at San Antonio approved this study, and the informed consent of all human subjects and their guardians was obtained. Inclusion criteria consisted of patients between 7 and 16 years of age with an immature permanent maxillary or mandibular single-rooted immature tooth with open apices (>2.5 mm of apical radiographic diameter) with a diagnosis of pulp necrosis with apical periodontitis with lesion or suppurative apical periodontitis and no systemic health problems that might interfere with healing of apical periodontitis (ie, immunocompromised, long-term steroid usage, etc.). A total of 12 patients were included in this study and consisted of 6 boys and 6 girls with an average age of 10.5 ± 2.7 years. Twelve teeth (1 per patient) were included in the study and were composed of 7 maxillary central incisors with history of complicated crown fracture and 5 mandibular premolars with history of dens evaginatus. All teeth were diagnosed with necrotic pulp and symptomatic apical periodontitis with radiographic evidence of a periapical lesion.

#### Regenerative Procedure and Sample Collection

The regenerative protocol consisted of the following procedures. Patients were anesthetized by using 1.8–3.6 mL of 3% mepivacaine without vasoconstrictor, with a buccal infiltration for maxillary teeth or an inferior alveolar nerve block for mandibular teeth. Subsequently, teeth were isolated with a rubber dam; caries, if present, was removed; and endodontic access was performed. The working length was determined radiographically by inserting #15 K-file size into the canal at the estimated preoperative canal length. The canals were disinfected with passive positive pressure irrigation with 20 mL of 6% NaOCl and 10 mL saline via a Max Probe (Dentsply Rinn, Elgin, IL) needle placed 1 mm from the radiographic apex of the tooth. The canals were then dried with paper points. The triple antibiotic paste consisted of USP grade antibiotic powders compounded in a 1:1:1 ratio of metronidazole, ciprofloxacin, and minocycline by a local pharmacy (Champs Biosystems, Foster City, CA) and mixed in double-distilled sterile water to form a paste-like consistency. A Food and Drug Administration Investigational New Drug permit was obtained for the off-label use of these antibiotics in this study. The antibiotic paste was delivered into the root canal spaces with the use of a Centrix syringe attached to a high-viscosity Accudose needle tube (Centrix, Shelton CT) under high-power magnification. The access was sealed with Cavit (3M, St Paul, MN).

Patients returned for the second treatment visit 1 month later. A positive blood aspirate (systemic blood sample) was collected in the cartridges during local anesthetic injection and immediately placed into an RNA isolation lysis buffer (RNeasy MiniKit; Qiagen, Valencia, CA). Teeth were isolated, accessed, and irrigated with sterile 0.9% saline; paper points were placed into the canal and allowed to absorb the saline intracanal samples for 2 minutes; the paper points were placed and removed under microscopic magnification to ensure that they did not touch the canal walls or the external surface. The paper points were immediately placed into RNA isolation lysis buffer (Qiagen). Bleeding from the apical region was achieved by rotating a pre-curved K-file size #25 at 3–5 mm beyond the working length. After microscope visualization of intracanal bleeding under high-power magnification, a second paper point was placed intracanal for 2 minutes to collect an intracanal blood sample, which was placed in RNA isolation lysis buffer (Qiagen).

#### RNA Isolation and Real-time Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated by using the RNeasy Mini Kit, and any potential genomic DNA contamination was removed by DNase treatment (DNA-free kit; Ambion, Austin, TX). Only RNA samples with an optical density A260/A280 ratio between 1.8 and 2.1 (indicative of RNA quality) were used (19). Therefore, if 1 or more RNA samples from a patient did not meet this prerequisite, the patient was excluded from the study. Of the total 12 sets of samples collected from 12 separate regenerative procedures, only 8 met this quality control inclusion criterion. The excluded samples were from 3 maxillary incisors with history of complicated crown fracture and from 1 mandibular premolar with history of dens evaginatus. Total RNA isolated from the included samples was used as template in 2-step real-time reverse-transcription polymerase chain reaction (RT-PCR) reactions. Initially, RNA templates (0.5 μg) were used in complementary DNA (cDNA) synthesis reactions by using Superscript reverse transcriptase II kit (Invitrogen, Carlsbad, CA). The cDNA templates were then used in real-time PCR reaction by using TaqMan Assays on Demand (Applied Biosystems, Foster City, CA) consisting of previously validated primers and probes specific for the mesenchymal stem cell markers: cluster of differentiation 105 (CD105) (aka endoglin gene assay #Hs00923997 g1) (20), cluster of differentiation 73 (CD73) (aka NT5E gene assay #Hs00159686 g1) (20), zinc finger and BTB domain containing protein 16 (ZBTB16) (assay #Hs00232313 m1), and cyclin D2 (assay #Hs00922419). In addition, the immune cell marker CD14 (assay #Hs02621496 s1), the dentinogenic/osteogenic marker dentin sialophosphoprotein (DSPP) (assay #Hs0171962) (21), the osteogenic marker alkaline phosphatase (ALK) (assay #Hs0346558 s1) (22), and an internal loading control (housekeeping gene) 18S (ribosomal 18S subunit gene; assay #Hs03005631 g1) were used. The reactions were run in triplicates of 25 μL, and cycle threshold (Ct) values obtained after the reactions were stopped at 45 amplification cycles. The Ct values for each target gene were normalized to the endogenous control (18S) values. The comparative delta-delta Ct method was used to normalize the data on the basis of the endogenous reference and to express it as the relative fold change in relation to the values obtained from the patient’s own systemic blood control levels after the exclusion criteria were verified by comparing primer efficiencies (23, 24).

#### Immunocytochemistry and Laser Confocal Microscopy

In addition to RNA isolation and real-time RT-PCR reactions, samples were also evaluated by immunocytochemistry. Intracanal blood samples were taken during treatment, and paper points were immediately placed in cold phosphate-buffered saline (PBS) buffer. The PBS-containing cells were pipetted onto glass slides and allowed to air dry. Samples were then fixed by submersion in 100% ETOH for 10 minutes, permeabilized, and blocked for nonspecific protein binding sites with blocking solution consisting of 4% normal goat serum (Sigma, St Louis, MO), 2% bovine gamma-globulin (Sigma), and 0.3% Triton X-100 (Fisher Scientific, Pittsburgh, PA) in PBS for 60 minutes before the incubation of primary antibodies consisting of an anti-CD105 (Endoglin, M3527; Dako, Carpinteria, CA; 1:500 dilution) raised in rabbit, anti-STRO-1 (MAB10; R&D Systems, Minneapolis, MN; 1:100 dilution) raised in mouse, or anti-CD73 (SC-101544; Santa Cruz Biotechnologies, Santa Clara, CA) raised in mouse. The slides were mounted and examined using a Nikon Microphot FXA microscope equipped for fluorescent visualization.
compared with the expression in systemic blood (Fig. 1). The expression of CD105 was increased compared with the systemic blood, the triggered bleeding into the root canal system in comparison to levels of mesenchymal stem cell markers observed in effect of tooth type on the expression of the genes evaluated. Collectively, riodontitis and consisted of 4 maxillary central incisors and 4 mandib-
were diagnosed as having pulpal necrosis and symptomatic apical pe-
 were rinsed in PBS, water, air-dried, and coverslipped with Vectashield (Vector Labs, Burlingame, CA), and immunoreactivity was visualized with a Nikon C1 si laser confocal imaging system equipped with 90i Nikon microscope (Nikon, Melville, NY).

Statistics
Gene expression data were analyzed with one-way analysis of vari-
ance with Bonferroni post hoc test, and significance was set at P < .05 by using the Graph Pad Prism 5.0 version software (Graph Pad, La Jolla, CA).

Results
Eight patients met the inclusion criteria and consisted of 5 boys and 3 girls with an average age of 9.3 ± 2.1 years. All teeth included were diagnosed as having pulp necrosis and symptomatic apical peri-
ondontitis and consisted of 4 maxillary central incisors and 4 mandibular premolars. Subset data analysis revealed no statistically significant effect of tooth type on the expression of the genes evaluated. Collectively, in comparison to levels of mesenchymal stem cell markers observed in the systemic blood, the triggered bleeding into the root canal system evoked a 178.9 ± 90.3 fold increase in CD73 (P < .05) and a 604.4 ± 262.8 increase in CD105 (P < .05) transcripts (Fig. 1A). Indeed, the systemic and intracanal saline samples demonstrated virtually no detectable expression of CD73. The expression of CD105 was increased 26.13 ± 4 fold (P < .05) in the intracanal saline sample when compared with the expression in systemic blood (Fig. 1A). Thus, the manipulation of periapical tissues and evoked-bleeding step that is a common step used in different regenerative protocols triggers a substantial influx of cells containing mRNA transcripts encoding mesenchymal stem cell marker genes into the root canal system.

We next evaluated whether the bleeding step triggers the influx of differentiated cells. The alkaline phosphatase gene (ALK-P) was not detected in any of the samples. However, the expression of DSPP was detected in 25% (2 of 8) of the intracanal blood samples, but its overall mean fold expression change (35.56 ± 25.3) was not statistically different when compared with the DSPP expression seen in systemic blood (Fig. 1B). The immune cell marker CD14 was detected in 25% (2 of 8) of the saline samples (106.6 ± 104.2 fold change; not statistically significant) and 50% (4 of 8) of the intracanal blood samples (14.39 ± 6.1 fold change; P < .05) (Fig. 1C). The ZBTB16 gene was detected in 50% (4 of 8) of the intracanal blood samples, but the overall expression change was not statistically significant when compared with the systemic blood levels. However, a significant up-regulation in the expression of the cyclin D2 gene was detected in 50% (4 of 8) of the saline samples (14.39 ± 6.1 fold change; P < .05), whereas no difference was detected in the expression of cyclin D2 in intracanal blood samples when compared with the systemic blood samples (Fig. 1D).

We next used immunocytochemistry to characterize the expression of stem cell markers within cellular profiles found in blood smears collected from the patient’s root canal system. The mesenchymal stem cell markers CD105, CD73, and STRO-1 were detected in cells collected from the canal space after the evoked-bleeding step in the confocal micrographs (Fig. 2A, B, and C, respectively).

Discussion
The involvement of stem cells in regenerative procedures has been hypothesized, but to the best of our knowledge, it has never been clinically demonstrated in patients. In the present study, we have demonstrated with real-time RT-PCR and immunocytochemistry that the evoked-bleeding step of a widely used regenerative endodontic treat-
ment protocol (13) results in the delivery of stem cells into root canal systems and that the levels of their molecular markers are up to several hundred fold greater than levels observed in the systemic blood collected from the same patients. The evoked-bleeding step involves the manipulation of the periapical tissues, and it is this manipulation step that appears to release stem cells from these tissues and results in their delivery to the canal system. Even though the exact source of these stem cells has yet to be determined, one likely source is from the apical papilla because it contains an enriched population of mesenchymal stem cells. However, these cells might be derived from several sources, including systemic blood or local tissues such as bone, peri-
odontal ligament, dental follicle, dental pulp, or the apical papilla (25–29). The data presented here not only demonstrate the delivery of stem cell markers into the root canal space, but they indicate that these cells most likely originate from local tissues adjacent to the apex of the root and not from the systemic circulation.

Mesenchymal stem cells, as stated by the Society of Cellular Therapy, must express the cluster of differentiation markers CD105, CD90, and CD73 in a population of cells (20). In addition, these markers have been used to isolate and clonally expand enriched mesen-
chymal stem cell cultures (30). Thus, we evaluated the expression of the CD105 and CD73 genes as a dependent measure of the relative presence of stem cells. We found that the expression of both CD105 and CD73 was substantially and significantly increased after the evoked bleeding from the periradicular tissues from immature teeth with open apices. These results are consistent with the hypothesis that the mechanical disruption of periradicular structures such as the apical papilla releases a substan-
tial quantity of stem cells from their niche. Thus, the blood invading the canal space after the evoked-bleeding step transports these cells to the target area (pulp canal space). It has been demonstrated that mesenchymal stem cells, probably bone marrow stem cells, can be found in the circulating blood (51). Therefore, we also collected systemic circu-

itary blood samples and normalized all the data to the expression level of each marker to levels observed in the patient’s systemic blood sample. Thus, the increased expression of mesenchymal stem cell markers is not due to the delivery of normally circulating stem cells, but instead it strongly implicates the local delivery of stem cells from the periradicular tissues into the root canal space. Interestingly, the expression or detection of the mesenchymal stem cell marker CD105 in samples collected during the saline flush before evoked bleeding suggests that a few stem cells were already detached or became detached from their niche after a gentle saline irrigation of the canal. It is possible that these cells could be surviving dental pulp stem cells from the apical pulp tissue that retained vitality despite advanced apical periodontitis, or these cells could have originated from the apical papillae or other periradicular structures such as periodontal ligament and bone.

The antibody STRO-1 was first characterized as a monoclonal anti-
body that recognizes a subpopulation of mesenchymal stem cells described as colony-forming units-fibroblasts because mesenchymal stem cells often resemble the morphology of fibroblasts in vitro (32, 33). Although STRO-1 has been widely used as a true mesenchymal stem cell marker, the gene that encodes this protein is unknown. Thus, only the protein expression of STRO-1, and not its corresponding mRNA transcript, could be evaluated in this study. Nonetheless, we have
used a well-characterized antibody against STRO-1 in addition to antibodies against CD105 to demonstrate, with the use of immunocytochemistry, that cells delivered after the evoked bleeding in regenerative procedures expressed the required CD105, CD73, and STRO-1 markers of mesenchymal stem cells. Also, immunohistochemical evaluation of these blood-delivered cells revealed that most cells had intact nuclei (visualized by TO-PRO-3 staining) and normal morphology that differed from other types of cells. Collectively, these findings suggest that cells positive for CD105, CD73, or STRO-1 were viable stem cells found in root canals after regenerative endodontic procedures.

Figure 1. Evoked-bleeding step in endodontic regenerative procedures in immature teeth with open apices leads to significant increase in expression of undifferentiated mesenchymal stem cell markers in the root canal space. Systemic blood, saline irrigation, and intracanal blood samples were collected during second visit of regenerative procedures. Real-time RT-PCR was performed by using RNA isolated from each sample as template, with validated specific primers for target genes and 18S ribosomal RNA endogenous control. (A) Expression of mesenchymal stem cell markers CD73 and CD105 was up-regulated after the evoked-bleeding step in regenerative procedures. (B) Expression of osteogenic marker ALK-P and dentinogenic marker DSPP was unaltered after the evoked-bleeding step in regenerative procedures. (C) Expression of innate immune response cell marker (CD14) was significantly detected at the second visit (saline sample) and after intracanal bleeding. (D) Differentiation marker ZBTB16 was not significantly changed during the regenerative procedure, whereas the expression of the proliferation marker cyclin D2 was significantly up-regulated in the saline irrigation sample before the evoked-bleeding step. Real-time RT-PCR was performed by using sample template. Data were normalized to the housekeeping gene 18S levels and presented as mean ± standard deviation fold increase in relation to systemic blood levels for each gene and analyzed with one-way analysis of variance with Bonferroni post hoc test (n = 8; *P < .05; **P < .01; n.s., not statistically significant).

The ALK-P and DSPP genes are known to be expressed in cells of osteoelastic and odontogenic cell lineages, respectively (34). Although DSPP is constitutively expressed in mature odontoblasts, its expression is significantly increased during the odontoblastic differentiation process (21). In the present study, the expression of these 2 genes was not significantly increased after regenerative procedures in immature teeth (only DSPP was detected in 2 of 8 samples). This finding suggests that the stem cells detected after regenerative procedures were undifferentiated cells and had not yet been committed to either osteoelastic or odontoblastic lineages.

The transcription factor ZBTB16 (aka promyelotic leukemia zinc finger transcription factor) gene is up-regulated during osteoblastic differentiation of stem cells because it is a transcription activator of the Runx2, collagen 1A, and alkaline phosphatase genes (35). In the present study, its expression was not detected in the immature teeth. This finding provides an additional line of independent evidence that the stem cells from the immature teeth were undifferentiated.
Cyclin D2 is a gene involved in cell cycle regulation, and its expression is detected and increased when stem cells leave the arrested stage and begin to proliferate during the Go/S cell-cycle transition (36, 37). We detected cyclin D2 in the saline samples of immature teeth (3 of 8 samples) but failed to detect a significant increase of cyclin D2 in the intracanal blood samples. It is possible that there were surviving stem cells already present in the root canal space undergoing proliferation before the evoked-bleeding step. Interestingly, the expression was not significantly increased in the samples after the intracanal-bleeding step. This might be due to the finding that stem cells are at an inhibited proliferation stage (arrested at Go) when in their niche (38, 39). Because samples were collected immediately after these cells were released from the manipulation of periapical tissues that might include the apical papillae (stem cell niches), we believe that transcription of the cyclin D2 gene had not yet been initiated.

CD14 is a marker for the innate response immune cells. It is highly expressed in macrophages, dendritic cells, and to a lesser extent in neutrophils (40). In this study, we detected a significant expression of CD14 in both saline (2 of 8) and intracanal bleeding (4 of 8) samples in immature teeth. However, because of the variability of the findings, only the intracanal bleeding sample was found to be statistically significant. This result is consistent with the prominent presence of innate response immune cells in apical periodontitis (41, 42). At the second visit after disinfection and triple antibiotic mix treatment, all patients had complete resolutions of symptoms (ie, no pain, sinus tracts, or swelling); therefore, the detection of the CD14 marker at the second visit is indicative of residual presence of neutrophils and macrophages.

One limitation of the present study is the inability to follow the cells over time. The real-time RT-PCR detection of these markers is merely a snapshot of the cells in time, and it represents the collective gene expression profile of the cells. The expression of genes and proteins in individual cells from this population of cells warrants more research. In addition, there is a myriad of genes involved in stem cell proliferation and differentiation that were not investigated in this study. One of the technical difficulties of this study is related to the very limited quantity of RNA sample collected in paper points. Also, only RNA samples that were of high quality with an optical density A260/A280 ratio between 1.8 and 2.1 (high quality RNA samples) were used. In the present study, 12 regenerative procedures were performed, but only samples from 8 regenerative procedures met this inclusion criterion and were included in the real-time RT-PCR experiments. Although we detected statistically significant differences in the expression of some of the genes, studies with larger sample sizes, perhaps multicenter studies, are warranted.

In conclusion, we have demonstrated for the first time that mesenchymal stem cells are delivered into root canal spaces during regenerative endodontic procedures in immature teeth with open apices. We believe that these findings provide the biological foundation for the involvement of stem cells in the continued root development and regenerative response that follow this clinically performed procedure. Further research is needed to evaluate how to increase the proliferative and differentiation capacity of these cells in the target tissues, the root canal space and the periapical environment. It is likely that the regeneration of pulpal tissues can be made even more predictable by the application of tissue engineering principles to use scaffolds, growth factors, and stem/progenitor cells to develop optimal and reliable endodontic regenerative procedures.

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The authors deny any conflicts of interest related to this study.
Clinical Research

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