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ABSTRACT

A major cause of apical periodontitis after endodontic treatment is the bacterial infiltration which could have been challenged by the presence of a vital pulp. In this study, self-assembled, scaffoldless, three-dimensional (3D) tissues were engineered from dental pulp cells (DPCs) and assessed as a device for pulp regeneration. These engineered tissues were placed into the canal space of human tooth root segments that were capped on one end with calcium phosphate cement, and the entire system was implanted subcutaneously into mice. Histological staining indicated that after three- and five-month implantations, tooth roots containing 3D scaffoldless engineered tissues maintained a cellular, fibrous tissue throughout, whereas empty tooth roots remained predominantly empty. Immunostaining indicated that the tissue found in the root canals containing scaffoldless DPC engineered tissues was vascular, as characterized by the expression of CD31, and contained odontoblast-like cells organized along the length of the root wall as assessed by immunostaining for dentin sialoprotein. This study shows that 3D self-assembled scaffoldless DPC engineered tissues can regenerate a vital dental pulp-like tissue in a tooth root canal system and are therefore promising for endodontic therapy.

KEY WORDS: tissue engineering, mesenchymal stem cells, calcium phosphate, regeneration, tooth, root canal.

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Scaffoldless Tissue-engineered Dental Pulp Cell Constructs for Endodontic Therapy

INTRODUCTION

Regenerative therapies for endodontics would prolong the life of the entire tooth organ. The dental pulp is comprised of vascular connective tissue with fibroblasts, blood vessels, nerves, and a population of stem cells that aid in the repair of the surrounding dentin (Liu *et al.*, 2006). Current endodontic treatments involve the replacement of infected dental pulp with an exogenous material. Although endodontic treatment has a high degree of success (Friedman *et al.*, 2003), one of the main causes for the future extraction of treated teeth is the formation of caries that potentially could have been repaired in healthy teeth by progenitor cells that reside in the pulp (Zadik *et al.*, 2008). Another common complication that necessitates the re-treatment of a tooth or tooth extraction is the re-introduction of bacteria into the pulp space through the deterioration of dental restorations. The presence of a vital pulp would provide a biological defense and maintain interstitial pulp pressure to deter such invasions (Torabinejad *et al.*, 1990; Ray and Trope, 1995; Stockton, 1999). The surrounding hard tissue of the pulp, the dentin, becomes mechanically altered after endodontic treatment, potentially due to the loss of moisture and interstitial pressure provided by the pulp tissue, which leads to higher fracture susceptibility in the tooth (Heyeraas and Berggreen, 1999; Akkayan and Gulmez, 2002; Soares *et al.*, 2007). Additionally, the generation of a vital dental pulp would facilitate continued root development in immature teeth. A tissue-engineered dental pulp could replace current methods of endodontic treatment and result in a physiologically functional pulp-dentin complex.

Several dental tissues contain populations of stem cells that could be used for regenerative therapies, such as the dental pulp from adult or deciduous teeth, apical papilla, or periodontal ligament (Gronthos *et al.*, 2000; Miura *et al.*, 2003; Seo *et al.*, 2004; Sonoyama *et al.*, 2008). Dental pulp stem cells are able to differentiate to form both dentin and pulp tissues, and therefore the combination of these cells with various scaffold materials is currently being investigated for use in endodontic treatments (Gronthos *et al.*, 2000). Commonly used scaffolding materials include calcium phosphates, collagen gels, or polymeric materials, due to their similarities with dentin or pulp tissues, and these systems have shown some success in the formation of dentin-like or pulp-like tissues (Gronthos *et al.*, 2000; Cordeiro *et al.*, 2008; Prescott *et al.*, 2008; Huang *et al.*, 2010). However, if cells regenerate a 3D tissue without the use of exogenous materials, the cells would potentially be able to

recapitulate native tissue more closely and generate their own preferred 3D microenvironment.

Recently, we have engineered self-assembled three-dimensional (3D) scaffoldless tissues from human dental pulp cells (DPCs). These samples are formed by culturing cells on dishes to generate cell sheets. These sheets lift from the substrate and are contracted by the cells toward 2 pins placed in the dish. The tissue sheet self-assembles into a cylindrical 3D, engineered tissue that is anchored to the plate by the pins. These 3D tissues are formed by the cells themselves without any exogenous materials; therefore, the cells are able to generate their own preferred microenvironment. Our previous studies have shown that these scaffoldless samples are highly cellular, solid tissues that express dentin proteins on the periphery and exhibit pulp properties in the core similar to those of the dentin-pulp complex in both *in vitro* culture and *in vivo* (Syed-Picard *et al.*, 2013).

The goal of this study was to investigate the use of scaffoldless 3D tissues engineered from DPCs as a mechanism to regenerate dental pulp-like tissues in the root canals of human teeth. This study assesses the potential of scaffoldless 3D tissues engineered by dental pulp cells for endodontic therapy.

MATERIALS & METHODS

Dental Pulp Cell Isolation

Healthy, adult third molars were obtained from the University of Pittsburgh, School of Dental Medicine, after routine extraction. All residual soft tissue was removed from the outer regions of the teeth, the teeth were fractured with a hammer, and the pulp was removed. The pulp was minced and then digested in an enzyme cocktail containing 3 mg/mL collagenase and 4 mg/mL dispase for 1.5 to 2 hr at 37°C. The total population of DPCs was plated and expanded in a growth medium (GM) containing Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA), with 20% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA, USA), and 1% penicillin/streptomycin (P/S; Gibco). Cells were used at passages 4 or 5.

Tooth Root Segment Preparation

Adult teeth were collected after extraction at the University of Pittsburgh, School of Dental Medicine. The crown regions were removed, and the roots were cut into radicular segments 5 to 7 mm in length by means of an IsoMet low-speed saw (Buehler, Lake Bluff, IL, USA) with an IsoMet diamond blade (Buehler). The root canal space was opened to a diameter of 1 to 1.5 mm (Figs. 1A, 1B). Root segments were then conditioned in a series of washes with NaOCl and EDTA, which has been previously shown to enhance DPC differentiation and adhesion to the dentin wall (Galler *et al.*, 2011). The roots were first soaked in 0.5 M ethylenediaminetetraacetic acid (EDTA) for 1 min, then rinsed in phosphate-buffered saline (PBS) for 5 min, and finally soaked in 6.15% NaOCl for 10 min. The roots were then washed 3 times in sterile PBS and then soaked again in 0.5 M EDTA for 10 min. The roots were again rinsed 3 times in PBS. To ensure sterility, the roots were kept incubated in GM at 37°C for 4 days and monitored for microbial growth. One end of each root was sealed with calcium phosphate cement (Fig. 1C).

Figure 1. Images of individual components of the root model and schematic of final construct. Images of tooth root samples from a coronal/apical and a proximal view, respectively (A, B), photograph of tooth root from a coronal/apical view with cement cap (C), image of 3D scaffoldless tissue engineered from DPCs (D), and schematic of final tooth root construct (E).

Formation and Delivery of DPCs in 3D Scaffoldless Engineered Tissues in/to Tooth Roots

Scaffoldless 3D samples were prepared from DPCs similarly to previously described methods (Syed-Picard *et al.*, 2013). Briefly, 35-mm-diameter tissue culture plastic dishes were first filled with Sylgard 184 silicone elastomer (Dow Corning, New York, NY, USA). The silicone was then coated with 3 $\mu\text{g}/\text{cm}^2$ mouse laminin (Invitrogen, Carlsbad, CA, USA). DPCs were plated onto sample dishes at a density of 200,000 cells/dish in GM with 5 mg/mL L-ascorbic acid (Fisher Scientific, Pittsburgh, PA, USA). At cell confluence, 2 minuten pins were placed in the center of each dish approximately 7 mm apart. Additionally, the culture medium was switched to one containing DMEM with 5% FBS, 1% P/S, and 2 ng/mL transforming growth factor beta 1 (TGF β 1). The tissue sheets were contracted by the cells to pull away from the edges of the dishes and roll toward the pins to form 3D scaffoldless tissues (Fig. 1D). From 2 to 4 samples were placed in the canal of the tooth root segment prior to implantation. A schematic of the final assembled construct can be seen in Fig. 1E.

Animal Implantation

All animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Prepared tooth root constructs containing scaffoldless DPC-engineered tissues

Figure 2. Empty tooth root constructs remain empty after three- and five-month implantations. Full image of H&E-stained section of empty sample after three-month implantation (A) and higher magnification images of the region near the open end of the root (B), center of the root canal (C), and cement seal (D). Full image of H&E-stained section of empty sample after five-month implantation (E) and higher magnification images of the region near the open end of the root (F), center of the root canal (G), and cement cap (H). Scale bars: A, E = 1 mm; B-D and F-H = 55 μ m.

within the canal or kept empty were implanted subcutaneously into immunocompromised Balb/C nude male mice (Charles River Laboratories, Wilmington, MA, USA); in total, 6 samples from each group were implanted. Incisions approximately 1 cm in length were made in the dorsal surface of the back, and pockets were created by blunt dissection. Four samples were implanted into each mouse, and samples were removed after either 3 or 5 mos.

Histology and Immunohistochemistry

After extraction, specimens were fixed in 10% formalin for 24 hr. Samples were decalcified in 0.32M EDTA for 2 to 3 wk, embedded in paraffin blocks, and sectioned longitudinally. Sections were stained with hematoxylin and eosin (H&E) or used for immunohistochemistry (IHC) with antibodies against

dentin sialoprotein (DSP) (LF151, provided by Dr. Larry Fisher, NIH) or CD31 (Abcam, Cambridge, MA, USA). IHC detection was performed with an EXPOSE IHC detection kit (Abcam).

RESULTS

In this study, scaffoldless tissues engineered from DPCs were inserted into the root canal space of tooth root segments for assessment of the regeneration of a dentin-pulp complex-like tissue. H&E staining of empty control samples after 3 and 5 months of implantation can be seen in Fig. 2. A small amount of host tissue infiltration is seen near the open end of the root and exhibits an adipose-like morphology, as seen in Figs. 2B and 2F at 3 and 5 months, respectively. However, the majority of the canal space remains empty (Figs. 2C, 2G). The calcium phosphate cement cap supported the infiltration of host cells (Figs. 2D, 2H). At 3 months, the spaces between the cement particles became highly cellular (Fig. 2D), and after 5 months, the infiltrated host tissue still remained in the cement region, but less cement is seen, potentially due to cement resorption (Fig. 2H).

Unlike the empty control samples, H&E staining shows that the canal spaces of root constructs containing 3D scaffoldless DPC samples are filled throughout with a vascular connective tissue after three- and five-month implantations (Fig. 3). These constructs contain an adipose-like tissue near the open end of the root (Figs. 3B, 3F) which is similar to the tissue seen near the open region of the empty tooth root constructs, and therefore is likely to be host mouse tissue. The center region of the canal space

is filled with a vascular connective tissue that is adhering to the dentin, and additional cells can also be seen lining the dentin wall (Figs. 3C, 3G). These cells were organized in a linear fashion and intimately associated with the dentin wall, similar to natural odontoblasts (Fig. 3C). The cement paste cap is filled with cells and tissue (Figs. 3D, 3H), similar to the empty tooth root samples; however, at 3 months, a dentin/bone-like tissue formed at the interface of the cement and the scaffoldless DPC tissue, indicating that the DPCs maintain the potential for hard-tissue generation (Fig. 3D), a characteristic of a native dental pulp. Immunostaining revealed dentin sialoprotein (DSP) expression, an indicator of odontoblast differentiation, at the dentin surface of root canals containing scaffoldless engineered tissues (Fig. 4A); this is not present in the empty control sample (Fig. 4B). This is similar to natural dental pulp, where odontoblasts line

the dentin wall of the pulp chamber. Additionally, immunostaining against CD31, an endothelial cell-surface marker, verified the presence of vascular tissue in the root canals of constructs containing scaffoldless DPC tissue (Fig. 4C). Analysis of these data, taken together, indicates that the scaffoldless DPC-engineered tissues facilitated the formation of a pulp-like tissue after being placed in a tooth root.

DISCUSSION

The regeneration of vital dental pulp tissue during endodontic treatment would prolong the life of the entire tooth organ. In this study, scaffoldless 3D tissues engineered from human DPCs were assessed for pulp regeneration. When inserted into a human tooth root, a dental pulp-like tissue was regenerated that was vascular, capable of forming dentin-/bone-like tissue, and containing odontoblast-like cells along the dentin surface, as seen by DSP expression. This study shows that 3D scaffoldless engineered tissues have potential for pulp-regenerative therapies.

Different tooth root models have been designed to study dental pulp regeneration (Gonçalves *et al.*, 2007; Cordeiro *et al.*, 2008; Huang, 2009; Huang *et al.*, 2010). The tooth root constructs used in the current study are based on the root fragment model described by Huang and colleagues (Huang, 2009; Huang *et al.*, 2010). The tooth root segments in the reported model were dimensionally similar to those used in our current study. The length of the segments ranged between 5 and 7 mm, and one end was sealed to mimic clinical conditions where vascular infiltration into the length of the root canal is accessible only at the apex. Huang *et al.* used mineral trioxide aggregate (MTA) to seal one end of the root segment, whereas a calcium phosphate cement was used as the capping material in our current study. Host tissue readily permeated the calcium phosphate cement; therefore, this end of the root contained more of a barrier than a seal. However, since the host tissue readily entered and filled the cement, a more biological seal was created. Ongoing studies are currently being performed by our research group to further investigate the use of this cement as a pulp-capping material.

In this study, DPCs were transplanted into the root canal spaces of human teeth *via* 3D engineered scaffoldless tissues. The cells secreted their own matrix to form an autogenous 3D

Figure 3. Tooth root constructs containing scaffoldless engineered dental pulp cell (DPC) tissues form a vascular connective tissue in root canals after three- and five-month implantations. Full image of H&E-stained section of tooth root construct containing scaffoldless DPC tissues after three-month implantation (A) and higher magnification images of region near the open end of the root (B), center of the root canal (C), and cement cap (D). Full image of H&E-stained section of tooth root constructs containing scaffoldless DPC tissues after five-month implantation (E) and higher magnification images of region near the open end of the root (F), center of the root canal (G), and cement cap (H). Black arrows show cells that are round in morphology and strongly adhering to the dentin, similar to odontoblast cells; arrowheads show dentin-/bone-like tissue, and asterisks indicate blood vessels. Scale bars: A, E = 1 mm; B-D, F-H = 60 μ m.

structure which could then be delivered to the root canal. Traditionally, cells are combined with exogenous materials before being transplanted into the body, to facilitate the formation of a 3D structure, help localize the cells to the region of regeneration, or direct cell behavior. Scaffolds for dental pulp engineering have been designed mainly from polymeric materials (Gonçalves *et al.*, 2007; Cordeiro *et al.*, 2008; Huang *et al.*, 2010) or from natural materials such as collagen or peptides (Prescott *et al.*, 2008; Galler *et al.*, 2012). However, exogenous materials are far from being able to recapitulate the multifaceted extracellular matrix scaffold that the cells generate for themselves. Other studies have been performed where DPCs were cultured in a scaffoldless pellet form and then implanted into amputated pulp in dogs (Iohara *et al.*, 2004). Pellet culture

Figure 4. Immunostaining indicates strong dentin sialoprotein (DSP) expression (brown) at the interface of dentin and engineered pulp tissue (black arrows) in tooth root constructs containing 3D scaffoldless dental pulp cells (DPC) tissues after three- and five-month implantations (A). This was not seen in the empty samples (B). Images are counterstained with hematoxylin (blue) to localize nuclei (A, B). Immunostaining also shows CD31 expression in tissues in tooth root constructs containing scaffoldless samples, indicating the presence of endothelial cells around blood vessels (arrowheads) after 3 and 5 months (C). Scale bars: A and B = 50 μ m; C = 85 μ m.

systems are formed by the centrifugation of cells to form aggregates. Although these systems are also considered to be scaffoldless, the cells are forced into a 3D structure by centrifugation, which exerts additional external forces onto the cells that can strongly affect cell behavior. In our self-assembled system, in addition to producing their own endogenous matrix, the cells arrange their own preferred 3D structure themselves, therefore fully controlling their environment and behavior. It has been hypothesized that the delivery of DPCs *via* aggregated cell

sheets, similar to the scaffoldless delivery methods used in the current study, would result in limited cell adherence to the root canal walls and may not support vascular infiltration (Murray *et al.*, 2007). However, in the current study it has been clearly shown that cells delivered *via* 3D scaffoldless engineered tissues do adhere to the root canal wall and express odontoblast markers, and that blood vessels can indeed penetrate the matrix.

Although it is known that the original engineered 3D tissue was generated by human DPSCs *in vitro*, it is unknown whether the regenerated dentin-pulp complex-like structure seen after *in vivo* implantation was produced exclusively by implanted human DPCs or with contributions from the host murine cells. The 3D engineered tissue could have acted as a scaffold that facilitated the infiltration of murine tissue. However, these results clearly show the feasibility of implanting a scaffoldless engineered tissue to regenerate a dentin-pulp complex. A mixture of host and implanted cells in humans would be an acceptable regenerative therapy.

This study shows that self-assembled 3D scaffoldless engineered tissues are a promising method to deliver cells into the root canal for pulp-regenerative therapies. The cells used in this study were isolated from adult third molars that were being discarded after routine extraction; this tissue could potentially be banked for future autologous therapeutic use. Further studies need to be performed to identify safe methods to expand autologous cells in a risk-free manner. The 3D scaffoldless DPC tissues described here could therefore be generated autologously without the introduction of foreign scaffold materials. This self-assembled scaffoldless system warrants additional evaluation for tissue replacement in pulpotomy models in larger animals to further test its clinical potential.

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