Dental Pulp Tissue Regeneration Using Dental Pulp Stem Cells Isolated and Expanded in Human Serum

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Abstract

Introduction: Dental pulp–derived stem cells (DPSCs) have the potential to regenerate dentin and dental pulp tissue because of their differentiation capacity and angiogenic properties. However, for regenerative approaches to gain regulatory and clinical acceptance, protocols are needed to determine more feasible ways to cultivate DPSCs, namely, without the use of xenogeneic-derived components (animal sera) and exogenous growth factors. **Methods:** In this study, human DPSCs were isolated from third molars and expanded in standard culture conditions containing fetal bovine serum (DPSCs-FBS) or conditions containing human serum (DPSCs-HS). After cell characterization and evaluation of their angiogenic secretome, DPSCs were seeded in tooth slice/scaffolds and implanted subcutaneously into immunodeficient mice. After 30 days, tooth slices were retrieved and evaluated for dental pulp tissue regeneration. Immunohistochemistry and confocal microscopy were used to quantify blood vessel formation and evaluate predentin and dentin formation. **Results:** After culture, DPSCs-HS produced concentrations of angiogenic growth factors equivalent to DPSCs-FBS. Additionally, in DPSCs-HS, several angiogenic factors were produced in at least 1-fold higher concentrations than in DPSCs-FBS. *In vivo*, it was determined that DPSCs-HS produced a robust angiogenic response and regeneration of dentin equivalent to DPSCs-FBS. **Conclusions:** These findings show that DPSCs can be isolated and expanded to clinical scale numbers in media devoid of animal serum or exogenous growth factors and still maintain their pulp regenerative properties. The implications of these findings are significant for further development of clinical protocols using DPSCs in cell therapies. *(J Endod 2016;■:1–7)*

Key Words

Angiogenesis, cell therapy, dental pulp stem cells, dentin, pulp tissue engineering

Significance

To our knowledge, this is the first study showing *in vivo* dental pulp tissue regeneration by adult dental pulp stem cells isolated and cultivated under conditions free of xenogeneic components, including animal serum and exogenous growth factors.

Affecting over 92% of adults, dental caries is globally the most prevalent chronic disease in both adults and children (http://www.nidcr.nih.gov/DataStatistics/FindDataByTopic/DentalCaries). Despite the array of restorative and endodontic modalities used to treat carious lesions, regenerating lost tooth structure and pulp tissue would be the ideal way to treat this disease. In the context of regenerative therapies, dental pulp–derived stem cells (DPSCs) provide promise for regenerating lost dental pulp and tooth tissue because of their ability to differentiate into odontoblasts and vascular cells to form pulplike tissues (1–9). A variety of protocols have emerged that describe different isolation and expansion methodologies for DPSCs. Although varying degrees of efficacy have been shown in these studies, a limitation to most current protocols is that the expansion of cells requires animal serum or the use of a number of exogenous growth factors. Before use in clinical applications, preclinical studies are needed that show that DPSCs can be predictably and efficiently procured under conditions that are more practical and cost-efficient for the clinical setting. We recently showed a clinically feasible means of tooth storage, DPSC isolation, and expansion of DPSCs without the use of animal serum (10). This study showed that saline was a suitable overnight storage media for third molars in order to isolate viable DPSCs using human serum instead of animal serum. However, the influence of these culture conditions on the capacity of DPSCs to regenerate pulp tissue was not determined *in vivo*.

The tooth slice model has been used to evaluate pulp regeneration using different regenerative approaches involving *in vivo* cell transplantation (11). Using this model, we have shown that *in vivo* transplantation of stem cells from exfoliated deciduous teeth (SHEDs) yields a robust angiogenic response and formation of dentin. As such, we used this model to determine if DPSCs isolated in conditions free of animal serum and exogenous growth factors, not only maintain their angiogenic capacity but also their *in vivo*

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capacity to regenerate pulp tissue. The hypothesis of the present study was that DPSCs expanded in human serum could regenerate dental pulp tissue in this *in vivo* model of dental pulp regeneration.

Methods

Tooth Storage and DPSC Isolation

Third molars were obtained from tooth extractions from patients ranging in age from 18–22 years and were placed in sterile saline solution according to the University of Michigan Institutional Review Board guidelines. The isolation of DPSCs was performed as previously described (2). Briefly, the crown of the tooth was cut just above the cementoenamel junction to open up the contents of the pulp. The pulp cells in the chamber and canals were cleaned out using various instruments, avoiding nerve tissue, and placed in Iscove modified Dulbecco medium (IMDM) without serum. After isolation, the cell suspension was placed in a conical tube and centrifuged at 1600 rpm for 5 minutes at room temperature. The supernatant was aspirated, and the pellet was resuspended in a 4 mg/ml dispase II/2 mg/ml collagenase solution. The solution was placed at 37°C for 60 minutes, inverting the tube at 15-minute intervals.

IMDM was added to the cells before the suspension was centrifuged at room temperature for 5 minutes at 1600 rpm. The cell pellet was resuspended in IMDM without serum. This cell suspension was placed in a T-25 tissue culture flask (Corning, Tewksbury, MA) containing 1 of the following: 15% fetal bovine serum + 100 uM ascorbic acid 2 phosphate + alpha minimum essential medium (α-MEM) (DPSCs-FBS) or 15% human serum + α-MEM (DPSCs-HS) (10).

Cell Proliferation/Population Doubling Time

Cells counts were performed at each passage, and the population doubling time (PDT) was calculated and compared between conditions. To determine the PDT, the following calculation was used:

$$\frac{(\#\text{ days from } P_0 \text{ to } P_1)(\log 2)}{(\log P_1 - \log P_0)}$$

where P_0 is the number of cells at the initial passage and P_1 is the number of cells at the next passage.

Cell Surface Marker Expression

Flow cytometry was performed to determine the expression levels of the cell surface markers CD90, CD73, CD105, and CD-45. DPSCs were harvested from T150 flasks (Corning), washed, and aliquoted equally into tubes. Cells were first incubated with a blocking solution containing CD16/CD32 at 4°C for 10 minutes followed by washing. Cells were then incubated with the specific antibodies conjugated with fluorochromes (Biolegends, San Diego, CA) at 4°C for 30 minutes. After washing, these cells were analyzed on a Beckman Coulter MoFlo flow cytometer (Beckman, Indianapolis, IN).

Angiogenesis Array

DPSCs were grown in either 15% FBS (DPSCs-FBS) or 15% human sera (DPSCs-HS) containing α-MEM. At passages 11 (DPSCs-FBS) and 12 (DPSCs-HS), cell layers were washed with serum-free α-MEM followed by the addition of 5 mL serum-free α-MEM before the cells were incubated at 37°C for 24 hours. After incubation, the cells and conditioned media were harvested and 5 ml of conditioned media was concentrated to 1.5 ml with Millipore Centrifugal Filters (Bedford, MA) and used for the angiogenesis array. Relative levels of 55 angiogenesis-related proteins were detected in this conditioned media using the Human Angiogenesis Antibody Array (Cat. #ARY007; R&D Systems, Minneapolis, MN). DNA concentration was measured from the harvested cells. Array membranes were exposed to x-ray film. These films were analyzed by quantifying the mean spot pixel densities using the freeware Image J software (http://rsb.info.nih.gov/ij; National Institutes of Health, Bethesda, MD). Samples were standardized using DNA concentrations.

Tooth Slice Model

The animal studies outlined conformed to the Animal Research: Reporting of *In Vivo* Experiments guidelines for animal research. To generate tooth slices, extracted noncarious third molars were used in accordance with the University of Michigan Institutional Review Board guidelines. After being extracted, the teeth were collected in a 0.1% sodium azide solution in phosphate-buffered saline. The periodontal soft tissue was removed with a periodontal scalpel. The molar was then fixed in a transversal sectioning apparatus at the pulp chamber level. With a diamond saw at low speed, a precision cut was made using refrigerated phosphate-buffered saline for cooling. One-millimeter-thick tooth slices were obtained, and pulp tissue was gently removed. A sodium chloride sieve (250–425 μm) was placed in the pulp chamber to be used as porogen. Medical-grade poly(L-lactide) (Resomer L 207 S; Boehringer Ingelheim, Ingelheim, Germany) with a molecular weight of 250,000 g/mol was dissolved in chloroform (5%) and dropped over the salt. After solvent volatilization, specimens were immersed in distilled water and stirred for 24 hours in order to obtain the highly porous poly(L-lactide) scaffold (11, 12).

DPSCs were cultured in either 15% fetal bovine serum (DPSCs-FBS) or 15% human serum (DPSCs-HS) containing α-MEM. At passage 5, DPSCs were harvested and resuspended in the appropriate media at a concentration of 6.7×10^7 cells/mL. Ten microliters of the cell suspension was mixed at a 1:1 ratio with Matrigel (Corning #354248) at 4°C. Approximately 20 μL of the cell-Matrigel suspension was transferred to the tooth slice. Tooth slices were incubated at 37°C for 60 minutes before being surgically implanted.

The tooth slices containing either DPSCs-FBS or DPSCs-HS were transplanted into the dorsal subcutaneous space of 6- to 7-week-old (~30 g) severe combined immunodeficient mice (n = 8) (CB.17 SCID; Charles River, Wilmington, MA). In 2 of the mice from each group, 3 intraperitoneal injections of 41.6 nmol/g body weight tetracycline hydrochloride (Sigma-Aldrich, St Louis, MO) were administered after 15, 20, and 25 days after surgery as shown (4). After 30 days, mice were euthanized, and implants were retrieved and fixed in 10% neutral buffered formaldehyde for 24 hours at 4°C until histologic processing was performed.

Histology and Immunohistochemistry: Vessel Formation

Vessel formation was evaluated as we have previously described (13, 14). Briefly, for total blood vessel counts, vessels were identified in hematoxylin-eosin–stained tissues at ×200 magnification by histologic structures with defined lumens and the presence of red blood cells within their boundaries. For defining vessels comprised of implanted human cells, paraffin-embedded tissue sections were deparaffinized in xylene, rehydrated, washed, and incubated in antigen retrieval solution (Dako Cytomation, Carpinteria, CA) before using a 1:100 dilution of a polyclonal rabbit anti–human factor VIII antibody (Thermo-Fisher-Lab Vision, Fremont, CA). Color development was performed with a Dako EnVision system kit (AEC, Dako Cytomation) according to the manufacturer's instructions. This staining protocol enabled the localization of the vessels formed by the implanted human DPSCs. The number of microvessels in 10 random fields per implant was counted under a light microscope (100×).

Confocal Laser Microscopy: Dentinogenesis

A confocal microscope (Leica SP5X; Leica Microsystems GmbH, Germany) with an ultraviolet laser (405 nm) was used to

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