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Stem/progenitor cells from inflamed human dental pulp retain tissue regeneration potential

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Abstract

Background—Potent stem/progenitor cells have been isolated from normal human dental pulps termed dental pulp stem cells (DPSCs). However, it is unknown whether these cells exist in inflamed pulps (IPs).

Aims—To determine whether DPSCs can be identified and isolated from IPs; and if they can be successfully cultured, whether they retain tissue regeneration potential *in vivo*.

Materials & methods—DPSCs from freshly collected normal pulps (NPs) and IPs were characterized *in vitro* and their tissue regeneration potential tested using an *in vivo* study model.

Results—The immunohistochemical analysis showed that IPs expressed higher levels of mesenchymal stem cell markers STRO-1, CD90, CD105 and CD146 compared with NPs ($p < 0.05$). Flow cytometry analysis showed that DPSCs from both NPs and IPs expressed moderate to high levels of CD146, stage-specific embryonic antigen-4, CD73 and CD166. Total population doubling of DPSCs-IPs (44.6 ± 2.9) was lower than that of DPSCs-NPs (58.9 ± 2.5) ($p < 0.05$), and DPSCs-IPs appeared to have a decreased osteo/dentinogenic potential compared with DPSCs-NPs based on the mineral deposition in cultures. Nonetheless, DPSCs-IPs formed pulp/dentin complexes similar to DPSCs-NPs when transplanted into immunocompromised mice.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

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Conclusion—DPSCs-IPs can be isolated and their mesenchymal stem cell marker profiles are similar to those from NPs. Although some stem cell properties of DPSCs-IPs were altered, cells from some samples remained potent in tissue regeneration *in vivo*.

Keywords

cytokine; dental pulp stem cell; IL- β ; immunocompromised mice; inflamed pulp; pulp/dentin complex; tissue regeneration; TNF- α

The role of stem/progenitor cells for cell-based therapy has been clearly recognized [1–4]. *Ex vivo*-expanded bone marrow-derived mesenchymal stem cells (BMMSCs) are being utilized for various clinical applications [5]. Many tissue-specific mesenchymal stem cells (MSCs) have also been identified, characterized and considered to be a potential cell source for tissue regeneration [6,7].

A type of cell in the dental pulp that may be viewed as similar to the osteoblast is the odontoblast. Although both types of cells are responsible for making hard tissues, odontoblasts are highly differentiated cells that no longer undergo self-replication. Additionally, the dentin they produce is structurally very different from bone, despite their chemical similarity. When odontoblasts are lost due to injuries to the tooth, they may be replaced by newly differentiated replacement odontoblasts derived from undifferentiated mesenchymal cells residing in the pulp. These undifferentiated mesenchymal cells are now considered dental pulp stem cells (DPSCs), which have been isolated and extensively characterized [8,9]. Using STRO-1 as a marker, cells in the pulp tissue immunoreacting to anti-STRO-1 antibodies are located within the perivascular cell population and express the pericyte marker 3G5, therefore, some of the pulpal pericytes are potentially DPSCs [10].

One striking feature of DPSCs is the formation of ectopic pulp/dentin complexes upon transplantation into immunocompromised mice [8]. Either heterogeneous populations or single colony (SC)-derived DPSCs are capable of generating this pulp/dentin complex. This observation leads to the possibility of utilizing DPSCs to regenerate pulp and dentin for clinical applications [11–15].

Another type of dental stem cell that is similar to the DPSC is stem cells from the apical papilla (SCAP), which have been proposed to be another great source for pulp/dentin regeneration [4,16,17]. Although DPSCs and SCAP are potent MSCs in terms of their self-renewal potential, the cells characterized by investigators so far have been isolated from extracted caries-free teeth, mostly third molars. These teeth contain a large volume of pulp tissue and therefore may give rise to a high number of DPSCs and SCAP for clinical applications. However, these type of teeth may not be available at the time of need and, furthermore, the SCAP only exist in immature teeth. Therefore, the source of these stem cells for pulp/dentin regeneration poses a problem.

Clinically, the entire pulp tissue is removed by pulpectomy as long as the tooth has been diagnosed with irreversible pulpitis even though a large portion of the pulp is still viable, in other words, the tissue is mildly to moderately inflamed or normal. It has not been investigated whether DPSCs isolated from teeth with irreversible pulpitis can exist and be isolated and still retain tissue regenerative potential. Within the skeletal system, inflammation can inhibit osteoblastic differentiation of human MSCs [18,19]. MSCs from arthritic joints have been shown to lose osteogenic potential [20]. The effect of inflammation within the dental pulp on DPSCs is not known. With the increasing interest in utilizing DPSCs for regeneration of dental tissues, we asked the following questions:

- Do DPSCs exist in inflamed pulps (IPs) and if yes, can they be isolated?

- Do isolated DPSCs from IPs show different stem cell properties as compared with those from normal pulps (NPs)?
- Will cytokines affect DPSC dentinogenic potency?
- Will isolated DPSCs from IPs retain tissue regeneration potential?

In this study, we systematically characterized DPSCs from IP and investigated their regenerative potential.

Materials & methods

Sample collection

The patient-related procedures utilized in this study conformed to the protocols approved by the Institutional Review Board of the University of Maryland-Baltimore College of Dental Surgery. NP tissue from impacted third molars without caries or pulp disease were collected as described previously [8,21]. IP tissue was collected from teeth diagnosed with irreversible pulpitis by pulpectomy. Pulps were extirpated, immediately placed in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC, USA) and frozen in -80°C for cryosectioning, or placed in culturing medium for cell isolation. Gingival tissues were collected from patients undergoing routine periodontal surgeries. All samples were obtained from generally healthy patients aged 14–22 years from the oral surgery, periodontics or endodontics department.

Cell cultures

Cells were isolated as described previously [8,14,21]. In brief, pulp tissues were minced and digested in a solution of 3 mg/ml of collagenase type I and 4 mg/ml dispase for 30–60 min at 37°C . Cell suspension was obtained by passing the digested tissue through a $70\text{-}\mu\text{m}$ cell strainer. The cells were pelleted and seeded in culture dishes, and incubated in α -minimum essential medium culture medium with 15% fetal bovine serum, 2 mM L-glutamine, $100\text{ }\mu\text{M}$ L-ascorbic acid-2-phosphate and antibiotics. After approximately 1–2 weeks, colony formation unit-fibroblasts (CFU-Fs) were formed. Stem cells from NP tissues were named DPSCs-NPs and from IPs DPSCs-IPs. In our pilot studies, DPSC-IP isolation had a low success rate of only approximately 30% either from contamination or no DPSC growth. Subsequently, only IPs of significant size were collected and sample collection medium was supplemented with antibiotics. We were then able to reach approximately 80% success, similar to DPSC isolation from NPs.

Multiple colony-derived DPSCs—Cells from the original colony pools were cultured and passaged at a 1:3 ratio when approximately 80% confluence was reached.

SC-derived DPSCs—Three to four colonies out of the multiple colony (MC)-derived DPSCs were randomly selected and subcloned using cloning rings. They were grown in separate wells and passaged in the same manner as the MC-derived DPSCs.

Healthy bone marrow aspirates from healthy volunteers were purchased from AllCells (Berkeley, CA, USA). BMMSCs were cultured based on our previous report [8].

Antibodies

For immunostaining, primary antibodies used were nonimmune mouse IgG control (Vector Laboratories, Burlingame, CA, USA), mouse monoclonal anti-human STRO-1 (Invitrogen Corporation, Carlsbad, CA, USA), mouse monoclonal anti-human CD90 (BD Biosciences, San Jose, CA, USA), mouse monoclonal anti-human CD105 (Abcam, Cambridge, MA,

USA) and mouse monoclonal anti-MUC18 (CD146; Invitrogen Corporation). For flow cytometric analysis, R-phycoerythrin (R-PE)-conjugated monoclonal anti-human antibodies to CD14, CD73, CD106, CD146 and CD166, and purified monoclonal anti-human antibodies to CD34, CD45 and CD105 were purchased from BD Bioscience. Anti-human stage-specific embryonic antigen (SSEA)-4 antibodies were purchased from Millipore (Temecula, CA, USA). Anti-human STRO-1 antibodies were kindly given by Stan Gronthos (Institute of Medical and Veterinary Science, Australia).

Immunohistochemistry & immunocytofluorescence

Frozen tissues were cryosectioned at 8 μ m and stained for STRO-1, CD90, CD105 and CD146. Sections were fixed with cold acetone at -20°C for 15 min, washed in phosphate-buffered saline (PBS), treated with 1.5% of hydrogen peroxide for 30 min, and washed and blocked with 5% normal goat serum (Vector Laboratories) or 2.5% normal horse serum (Vectastain Elite ABC kit; Vector Laboratories) for 1 h. Sections were then incubated with primary antibodies for 1 h at room temperature. Primary antibodies were mixed with 1% bovine serum albumin to the following dilutions: STRO-1, 1:25; CD90, 1:10; CD105, 1:20; CD146, 1:50. They were then washed and the appropriate secondary antibodies added for 1 h. Secondary antibodies used were biotinylated goat anti-mouse IgM antibody (Vector Laboratories) or IgG antibody. After washing, avidin–peroxidase complex was added and incubated for 30 min followed by washing and the addition of peroxidase substrate solution for 5 min. Sections were counterstained with Mayer's hematoxylin solution (Sigma-Aldrich, St Louis, MO, USA) and mounted in aqueous mounting medium (Biomedica, Foster City, CA, USA). For the negative control sections, nonimmune antibodies were used.

For immunocytofluorescence, cells at subconfluence in chamber slides were fixed in 100% ice cold methanol and incubated in blocking buffer (32.5 mM NaCl, 3.3 mM Na_2HPO_4 , 0.76 mM KH_2PO_4 , 1.9 mM NaN_3 , 0.1% (w/v) bovine serum albumin, 0.2% (v/v) Triton-X 100, 0.05% (v/v) Tween[®] 20 and 5% goat serum) for 30 min followed by addition of monoclonal mouse anti-human STRO-1 antibodies for 1 h at room temperature. After washing, cultures were incubated with anti-mouse Alexa Fluor 488 for 1 h at room temperature and the cell nuclei stained with DAPI (Invitrogen). Images were analyzed under a fluorescence microscope.

Population doubling

Dental pulp stem cells were seeded at low density (~ 60 cells/ cm^2) and allowed to grow until approximately 70–80% confluence. Cells were then passaged at the same cell density. The population doubling (PD) was calculated at every passage based on our previous report [22]. To determine finite PDs, cumulative addition of total numbers were generated from each passage until the cells ceased dividing [23]. The criteria for cell senescence was that the cells did not divide for a month in culture and that over 60% of the cells were stained positive for β -galactosidase [24].

To determine the effect of cytokine on PDs, DPSCs-NPs from each donor ($n = 6$) were divided into two groups. Nonstimulated control cells and cytokine-stimulated cells underwent the same process for PD studies. Cultured cells at passage 2 in culture medium or stimulated with 10 ng/ml of TNF- α (Cell Signaling Technology, Danvers, MA, USA) and 20 ng/ml of IL-1 β (eBioscience, San Diego, CA, USA) for 48 h before they were passaged for PD studies. The two cytokines were continuously present for the stimulated group until the end of the PD studies.

Immunophenotype analysis

Cells at passage 3 (1×10^5) were stained with R-PE-conjugated antibodies against cell surface marker antigens (CD14, CD73, CD146 and CD166) or with purified antibodies against cell surface marker antigens (STRO-1, CD34, CD45 and SSEA-4), followed by incubation with R-PE-conjugated secondary antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL, USA). Subclass-matched antibodies were used as controls. DPSCs-NPs and BMSCs were also tested as comparisons. They were analyzed on a FACSCalibur flow cytometer (BD Bioscience). Positive cells were defined as the level of fluorescence more than 99% of the corresponding isotype-matched antibodies (BD Bioscience).

Differentiation induction

Cells undergoing differentiation stimulation were seeded onto 12- or 48-well plates at $0.5\text{--}1 \times 10^4$ cells/cm². Subconfluent cultures were incubated for 2 weeks in osteo/dentinogenic, adipogenic or neurogenic medium (Table 1). Media were changed every 2–3 days. At the end of differentiation stimulation, cells were analyzed with either chemical staining, or harvested for RNA isolation for real-time reverse-transcription-PCR (qRT-PCR) to determine the lineage-specific gene-expression profile. The primers used for PCR are listed in Table 2.

For osteo/dentinogenic differentiation, after 2 weeks, cells were stained with Alizarin red S (Sigma) for the detection of mineralized matrix as described previously [8,14,22]. For adipogenic differentiation, after 2 or 6 weeks of stimulation, cells were fixed in 10% formalin for Oil Red O staining as described previously [14]. The cytokine effects on differentiation were performed with the presence of 10 ng/ml of TNF- α and 10 ng/ml of IL-1 β prechallenge for 48 h. The concentrations of the cytokines were determined based on reports that demonstrated observable biological effects under these concentrations [25–27], although physiological concentrations of these cytokines may not reach this level *in vivo* [28–30]. The two cytokines were continuously present during the course of induction.

qRT-PCR

Total cellular RNA was isolated using an easy RNeasy mini kit (Qiagen, Valencia, CA, USA) with DNase I (Invitrogen) to remove genomic DNA contaminant. The extracted RNA was reverse transcribed to generate the first strand cDNA using Superscript II (Invitrogen). The produced cDNA was used as a template for each PCR reaction using IQ SYBR Green Super Master Mix (Bio-Rad, Hercules, CA, USA). The PCR reaction was performed using ABI 7300 with the following thermal cycling condition: 50°C for 2 min, 95°C for 2 min, 95°C for 15 s, 60°C for 1 min, cycled to step three for 40 cycles. The resulting PCR products were run on a 4% agarose gel with ethidium bromide (Invitrogen) and gel images were captured and quantified with Kodak Gel Capture 2300 image documentation system. PCR reactions were performed using the human-specific sense and antisense primers, which are designed according to published cDNA Genbank sequences (Table 2). A relative quantitative analysis method using the standard curve was performed to quantify the relative gene expression compared with the level of the housekeeping gene *GAPDH*.

In vivo transplantation

Cells at passage 3 (4×10^6) were mixed with the carrier hydroxyapatite and tricalcium phosphate powder (40 mg, Zimmer Inc., Warsaw, IN, USA) and incubated for 90 min at 37°C. The mixtures were subcutaneously transplanted into Begie XIDIII nu/nu mice (female, 8–12 weeks old; Harlan Sprague Dawley Inc., Indianapolis, IN, USA) [31]. A total of 8 weeks after the implantation, the resected tissues were fixed with 4% paraformaldehyde in PBS (pH 7.2) and decalcified with 5% ethylenediaminetetraacetic acid in PBS (pH 7.2)

for 2 weeks. The samples were dehydrated, embedded in paraffin blocks and sectioned (6 μm) for hematoxylin and eosin staining. Under the microscope, seven fields were randomly selected for each sample section and the total dentin-like mineralized tissues within each field were counted and analyzed by ImageJ.

Data analysis

Kruskal–Wallis analysis of variance (ANOVA) by ranks was used to compare DPSC densities in NPs/IPs. This nonparametric test was used because ANOVA's assumption of independence between experimental groups was not met. Kruskal–Wallis's power was virtually equal to that of ANOVA (95.5% of ANOVA's power). Tukey's honestly significant difference (HSD) test was used to distinguish significant differences between groups. A *t*-test was used when only two groups (PD and *in vivo* studies) were compared. A $p \leq 0.05$ was considered significant.

Results

Expression of gene markers in IPs

A list of markers that may be used to identify MSCs has been proposed [32]. We selected several markers that have been shown to be expressed by isolated MSCs in cultures, including STRO-1, CD90, CD105 and CD146, and determined whether their expression could be detected in IPs. NPs were used as a comparison. Immunoreactivities of these antigen markers with the antibodies were clearly observed in pulp tissues as demonstrated in Figure 1. All four markers appeared to be located within the vascular or nerve structures. In IPs, calcified aggregates in circular forms were frequently observed and appeared to be disrupting the vascular structures. Because of inflammation, IP samples showed more disorganized structures that are reflected by the different appearance of the stained structures from those in NP samples. In addition to staining that was associated with structures, a few scattered fibroblast-like single cells were also immunoreactive to the antibodies. Gingival tissue was used as a negative control. No staining was observed with STRO-1 in gingival tissues (Figure 1). However, the other three markers were all expressed in the lamina propria with a greater density for CD90 than CD146 and CD105. The expression was also associated with vascular structures. No staining was observed within the epithelium for any of the four markers.

We next determined the densities of the staining comparing IPs and NPs. The stained loci count was performed at x400 magnification under the microscope for three randomly selected areas per sample section. Stained loci densities of STRO-1, CD90, CD105 and CD146 were all significantly higher in IPs than in NPs ($p < 0.05$, Table 3).

Clonogenic primary cultures of heterogeneous stem/progenitor cells from IPs

The ability to form CFU-Fs is one of the important characteristics for MSCs. The DPSC-CFU-Fs normally present with great variation in their morphologies (e.g., cell density) within the colonies as we have described before [8,21]. A similar phenomenon was also observed from CFU-Fs derived from IPs, as shown in Figure 2. Furthermore, when subcloning the cell colonies, variation among clones was also noted, which is typical of heterogeneous populations of primary stem/progenitor cells without specific subpopulation selections. These cells hereof are termed DPSCs-IPs in order to conform to the previously termed DPSCs derived from NPs [8]. Some cell colony clones from DPSCs-IPs appeared to be very robust in terms of proliferative capacities, as indicated in Figure 2E. Colonies of high cell density were observed. The DPSCs-IP expressed STRO-1 in cell culture (~10%) (Figure 2).

DPSCs-IPs showed lower PD than DPSCs-NPs

To determine whether DPSCs-IPs have a similar capacity to self-renew and expand *in vitro*, PD studies were carried out for both MC and SC cells. The results are summarized in Table 4 & Figure 2G. The total PD of cultured MC DPSCs-IPs (mean $\pm$ standard error of the mean: 44.6 ± 2.9) was significantly lower statistically than that of MC DPSCs-NPs (58.9 ± 2.5 ; $p = 0.003$). Significant difference also existed between SC DPSCs-NPs and SC DPSCs-IPs ($p = 0.025$). Owing to the wide variation of PD of SC DPSCs-NPs, no significant difference was detected between MC and SC DPSCs-NPs ($p = 0.11$), while a significant difference was found between MC and SC DPSCs-IPs ($p = 0.003$). Cytokine treatment appeared to lower the PD of DPSCs-NPs ($p = 0.005$). DPSCs (NPs and IPs) after up to ten passages using the method of passaging described in the 'Materials & methods' section underwent senescence, which was verified by detecting the senescence-associated β -galactosidase activity (Figure 2H). Close to 100% of cells were positive of staining in all cultures at the end of the PD studies.

DPSCs-IPs exhibited similar immunophenotype to DPSCs-NPs & BMMSCs

Multiple colony-derived DPSCs-IPs (samples #2 and 3) and DPSCs-NPs as well as BMMSCs were subjected to flow cytometry analysis for the detection of cell surface markers. As shown in Figure 3, STRO-1, CD73, CD146, CD166 and SSEA-4 were notably positive on all cells tested. Hematopoietic markers CD34, CD45 and CD14 were basically not expressed. Interestingly, the embryonic stem cell marker SSEA-4 was significantly expressed in DPSCs-IPs and -NPs compared with BMMSCs. STRO-1 expression by DPSCs-IP sample #2 at passage 3 reached approximately 22%, indicating that some IPs can harbor stem cells.

Inflammation may affect the outcome of cell differentiation

To determine whether the stem cell properties of DPSCs in IPs were affected, the following approaches were undertaken:

- DPSCs-IPs were stimulated with differentiation media and examined for the gene expression of specific lineages compared with DPSCs-NPs under the same differentiation stimulation for 2 weeks;
- DPSCs-NPs were stimulated with differentiation media in the presence of cytokines (TNF- α plus IL-1 β) for 2 weeks to determine whether cytokines affect DPSC differentiation.

Three differentiation pathways, osteo/dentinogenic, adipogenic and neurogenic, were tested.

Osteo/dentinogenic differentiation studies—The mineralization appeared to be affected by the presence of cytokines and some DPSCs-IPs may have less capacity to accumulate these deposits (Figure 4A). When osteo/dentinogenic gene markers were examined by qRT-PCR, the baseline gene-expression levels of some markers were already higher than the induced (*DSPP*, *BSP*, *ALP* in DPSCs-NPs). DPSCs-IPs, however, had lower baseline levels of all gene markers examined compared with the induced levels. Individual gene-expression levels appeared to be affected by the presence of cytokines differently (Figure 4B).

Adipogenic differentiation studies—The presence of cytokines appeared to have downregulatory effects on the two adipogenic markers examined. DPSCs-IPs appear to have a lower capacity for adipogenesis as the two marker genes (*LPL* and *PPAR γ 2*) were not as upregulated as in DPSCs-NPs, especially *LPL* (Figure 4B). The Oil red O stain was minimal to none in all DPSC cultures examined. This conforms to a previous finding that DPSCs are

not potent in adipogenesis [14,17]. A few DPSCs-NPs showed accumulation of oil droplets after 6 weeks of adipogenic stimulation but none was detectable in DPSCs-IPs (Figure 4B).

Neurogenic differentiation studies—The qRT-PCR demonstrated that the expression of β III-tubulin and *NFM* were reduced in the presence of cytokines (Figure 4B). DPSCs underwent morphologic transformation with elongated cellular processes after differentiation induction (data not shown).

***In vivo* pulp/dentin complex formation**

The tissue regenerative capacity of DPSCs-IPs was determined using the subcutaneous model of immunocompromised mice as described in our previous reports [8]. DPSCs from NPs and IPs either MS or SC were transplanted into the mice for 8 weeks. Histological examination revealed that DPSCs-NPs formed mineralized dentin-like tissue deposited perpendicularly against the hydroxyapatite and tricalcium phosphate carrier. This dentin-like tissue also contained densely ordered collagen matrix with odontoblast-like cells linings. The mineralized matrix surrounded dense connective tissue supplied with blood vessels resembling the dental pulp, and forming a pulp/dentin complex (Figure 5). DPSCs-IPs also formed pulp/dentin-like complexes. There was great variation in the amount of mineralized tissue formed by the cells.

Dental pulp stem cells from inflamed dental pulp (#1) formed low amounts of mineralized tissues and no pulp/dentin complexes were observed. DPSCs-IPs (#2), however, were able to establish good pulp/dentin complexes either derived from MC or SC, except SC-4a (Figure 5D). Dense connective pulp-like tissue was surrounded by dentin-like structures and was supplied with blood vessels. These complexes were similar to those formed by DPSCs-NPs. DPSCs-IPs (#3) either MC or SC were also capable of forming pulp/dentin complexes but to a lesser extent than DPSCs-IPs from sample #2.

Discussion

From our data we can answer the questions we raised earlier and conclude the following:

- DPSCs exist in IPs and can be isolated
- There is a difference between DPSCs from IPs and NPs
- Cytokines affect dentinogenic potency of DPSCs *in vitro*
- DPSCs-IPs retain tissue regeneration potential *in vivo*

Although DPSCs-IPs appear to lose some stem cell properties based on the *in vitro* assays, they still demonstrate the capacity to form a pulp/dentin complex *in vivo*. From the perspective of clinical applications, DPSCs-IPs may potentially be used as a cell source for pulp and dentin regeneration. To date, pulp tissues removed by pulpectomy due to inflammation are discarded as medical waste. Our findings may provide an alternative use of this discarded tissue.

Our *in vitro* assays demonstrated that DPSCs-IPs have slightly lower PDs than their normal counterparts. This indicates that inflammation may affect the total number of cell divisions. Because the potent proinflammatory cytokines TNF- α and IL-1 β are upregulated in the inflamed sites, they may act on local stem cells. We utilized an *in vitro* system to determine whether these two cytokines affect the PDs of DPSCs-NPs. The results suggest that direct exposure of cells to the cytokines appears to cause the cells to senesce more prematurely, which corresponds to the finding of lower PDs of DPSCs-IPs than DPSCs-NPs. In terms of cell surface marker profiles, isolated DPSCs-IPs express similar amounts to DPSCs-NPs

(Figure 3). The tested DPSCs-IPs have a higher or equal level of STRO-1, CD146, CD73, CD166 and SSEA-4 compared with DPSCs-NPs or BMMSCs. Interestingly, the expression of the embryonic stem cell marker SSEA-4 is higher in DPSCs than BMMSCs. DPSCs-IPs from sample #1 expressed higher levels of STRO-1 than other cells tested. Several embryonic stem cell associated genes such as *Oct4*, *Nanog* and *Rex-1* are expressed in DPSCs [33], suggesting that these cells are highly robust as stem cells.

All four MSC markers STRO-1, CD90, CD105 and CD146 examined *in situ* were expressed in both NPs and IPs. Basically, the staining locations are associated with vascular structures and some may be associated with neurosheath [10]. Further studies are needed to verify this association by co-labeling with anti-CD31 or anti-CD34 antibodies. IPs expressed greater amounts of all four markers compared with NPs. This may be due to the higher vascularity that occurred during inflammation and these markers are all associated with blood vessels. It was reported that TNF- α and IL-1 α upregulate VEGF in pulp, which is an angiogenic factor [34]. This may explain why more staining was observed in IPs. Both CD105 and CD146 are also expressed by endothelial cell lineages, therefore, their expression in pulps does not exclusively represent the existence of DPSCs. The flow cytometry data revealed a high expression level of CD146 of DPSCs. Further studies are needed to understand the physiological significance of these subpopulations of DPSCs in pulps.

Since DPSCs-IPs and cytokine-stimulated DPSCs-NPs showed lower PDs, it is possible that cytokines may affect the differentiation capacities of DPSCs-NPs and the DPSCs-IPs may already have less multilineage differentiation potential. For the osteo/dentinogenic pathway, we found that the presence of cytokines interfered with the mineralization. DPSCs-IPs in cultures without the presence of cytokines already showed lower mineral deposits in most samples tested. While the calcium accumulation was clearly reduced with the presence of cytokines, the marker gene-expression profiles were more complex. The expression of *ALP* and *OCN* after differentiation induction appeared to be affected (reduced expression), whereas *DSPP*, *BSP* and *CBFA1* were not. These data indicate that these marker genes are affected by cytokines in different ways. Nonetheless, the end effect of osteo/dentinogenesis was affected by the presence of cytokines (i.e., reduction of mineralization) and this clearly interfered with the function of osteo/dentinogenic differentiation. Reports have shown that TNF- α inhibits differentiation of osteoblasts from precursors as shown by reduced formation of multilayered, mineralizing nodules and decreased secretion of the skeletal-specific matrix protein OCN. TNF- α , however, does not affect the expression of the osteogenic bone morphogenic proteins (BMP-2, -4 and -6), or skeletal LIM protein-1 [35]. TNF- α inhibits CBFA1 activation by β -glycerophosphate [36]. Both *ALP* and *OCN* were downregulated in DPSCs by cytokines in our current studies, which parallel the findings for osteoblast lineages mentioned earlier, although the expression of *DSPP*, *BSP* and *CBFA1* was not negatively affected by cytokines.

Under normal *in vivo* environment, DPSCs have not been demonstrated to differentiate into adipocytes or neural cells; however, they have the capacity *in vitro* under a controlled environmental setting. Therefore, the capacity of DPSCs to differentiate *in vitro* was utilized to study the effect inflammation has on this differentiation. A diminished capacity to differentiate as a result of inflammation or cytokine stimulation suggests the loss of stem cell properties. Adipogenic differentiation potential also appears to be reduced by cytokines. Among the markers examined for neurogenic differentiation, the expression of β III-tubulin and *NFM* was downregulated by cytokines, whereas the expression of *nestin* and *CNPase* was not. The high baseline expression may indicate that a minority of uninduced cells underwent spontaneous differentiation and the signals were amplified during PCR procedure.

Although our data indicated that inflammation reduced the capacity of DPSCs to differentiate, most DPSCs-IPs tested remained capable of tissue regeneration. The amount of cytokines used in our *in vitro* assay was high, which may not occur *in vivo*. In addition, other cytokines, such as IL-6, found in the inflamed site may increase MSC stemness [37]. *Ex vivo*-expanded MSCs are also well known to have immunosuppressive and anti-inflammatory properties *in vitro* and when implanted into *in vivo* models [38,39]. Therefore, DPSCs isolated from IPs may retain their stem cell potency as suggested in our *in vivo* findings (Figure 5). It was frequently observed that calcified masses and aggregates are scattered throughout the IP tissues [40]. They appear to destroy blood vessels and eventually disrupt the pulp architecture (Figure 1). These aggregates may interfere with the pulp self-regeneration because they are unlikely to be resolved. Therefore, the IP tissues may be removed from the canal and DPSCs isolated. These cells can be expanded *in vitro* and replanted back in the canal carried by scaffolds for pulp/dentin regeneration, either for the same tooth or for other teeth of the same host. As mentioned before, third molars or other types of teeth in pristine condition as a source of autologous DPSCs is relatively limited. Our findings provide an alternative and perhaps more attainable source of DPSCs, thereby increasing opportunities for cell-based therapy to regenerate pulp/dentin tissues.

Two findings from this study require further investigation. First, DPSCs from IPs have altered stem cell properties. The molecular mechanisms underlying the changes in cell properties require further study in order to determine whether their properties can be reversed to those of DPSCs-NPs. Second, DPSCs-IPs retain the potential to regenerate tissues. Additional studies are needed to establish a protocol to verify the consistency of the tissue regeneration potential of DPSCs-IPs; for example, whether subpopulations of DPSCs-IPs should be isolated to achieve higher quality tissue regeneration.

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Figure 1. Immunohistochemical analysis of mesenchymal stem cell markers STRO-1, CD90, CD105 and CD146 in normal pulps (A–H), inflamed pulps (I–P) and gingival tissues (Q–T) Red arrowheads point at representative brown staining associated with vascular structures. Green arrowheads indicate the mineral particles accumulated in the lumen of blood vessels. In negative controls, no detectable stain was observed (not shown). Scale bars: (A–D, I–L & Q–T) 100 μ m; (E–H & M–P) 30 μ m.

Figure 2. Clonogenic dental pulp stem cells in cultures and population doubling studies
(A) DPSCs-NP #2 from a 9-year-old female, tooth #6 at passage 0. **(B)** DPSCs-IP #1 from a 17-year-old male, tooth #19 at passage 4. **(C)** DPSCs-IP #2 from a 19-year-old male, tooth #30 at passage 0. **(D)** DPSCs-IP #3 from a 15-year-old male, tooth #30 at passage 0. **(E)** DPSCs-IP #2 SC-5a from a 19-year-old male, tooth #30 at passage 2. Cell fixed and stained with toluidine blue. **(Fi)** STRO-1 fluorescence staining (green) and DAPI nuclear staining (blue) of seeded DPSCs-IP #3 MC from a 15-year-old male, tooth #30 at passage 2. **(Fii)** Nonimmunized isotype control of the same cells in **(Fi)**. Scale bars: **(A–D)** 200 μ m; **(E)** 1 mm; **(F)** 20 μ m. **(G)** Relative population doublings between DPSCs-NPs, cytokine (TNF- α plus IL-1 β) treated DPSCs-NPs and DPSC-IPs. *Significant difference ($p \leq 0.005$); error bars: standard error of the mean.
(H) β -galactosidase expression of senesced DPSCs at the end of population doubling studies (no β -galactosidase staining was observed at low passage of DPSCs, data not shown).
 DPSC: Dental pulp stem cell; IP: Inflamed pulp; MC: Multiple colony; NP: Normal pulp; SC: Single colony.

Figure 3. Immunophenotype analysis of human dental pulp stem cells and bone marrow-derived mesenchymal stem cells by flow cytometry

Multiple colony-derived cells (1×10^5) at passage 3 were incubated with specific monoclonal antibodies against cell surface marker antigens STRO-1, CD73, CD146, CD166, SSEA-4, CD14, CD34 and CD45 followed by secondary antibodies (R-phycoerythrin). Positive signals are indicated by the red area. Subclass-matched control antibodies were used for the controls (white area). M1 represents fluorescence intensity exceeding 99% of the controls. BMMSCs were used for comparison. DPSCs-IPs sample #2: DPSCs-IP from a 19-year-old male, tooth #30; DPSCs-IP sample #3: DPSCs-IP from a 15-year-old male, tooth #30.

BMMSC: Bone marrow-derived mesenchymal stem cell; DPSC: Dental pulp stem cell; FI: Fluorescence intensity; IP: Inflamed pulp; NP: Normal pulp; SSEA: Stage-specific embryonic antigen.

Figure 4. Differentiation induction analysis

DPSC-NP or DPSC-IP cultures from different samples indicated by numbers stimulated with induction media with or without the presence of cytokines for 2 weeks. Cultures in the wells stained with Alizarin red (A) or cells harvested for real-time reverse transcription-PCR analysis (B). The bar charts represent the relative expression levels of each gene normalized against *GAPDH*. DPSCs-NPs (n = 3), DPSCs-IPs (n = 2); error bars: mean $\pm$ standard error of the mean. Images under the adipo real-time PCR bar chart panel are representative data of Oil Red O staining of a few DPSCs-NPs with minimal oil droplet accumulation intracellularly after 6 weeks of adipogenic stimulation. No Oil Red O stain was observed in stimulated DPSC-IP cultures. Scale bar: 25 μ m. ALP: Alkaline phosphatase; BSP: Bone sialoprotein; CBFA1/RUNX2: Core-binding factor- α 1; CNPase: 2'3' cyclic nucleotide 3' phosphodiesterase; Cyto: TNF- α plus IL-1 β presence in cultures during the entire incubation periods; Diff: Differentiation induction; DPSC: Dental pulp stem cell; DSPP: Dentin sialophosphoprotein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IP: Inflamed pulp; LPL: Lipoprotein lipase; NFM: Neurofilament M; NP: Normal pulp; OCN: Osteocalcin; PPAR γ 2: Peroxisome proliferating activated receptor γ 2.

Figure 5. *In vivo* formation of pulp/dentin complexes by transplanted dental pulp stem cells Cells mixed with HA/tricalcium phosphate were subcutaneously transplanted into Begie XIDIII nu/nu mice. After 8 weeks, the resected tissues were processed for hematoxylin and eosin staining. **(A)** Dental pulp stem cells (DPSCs) from normal pulps as the control. **(B)** DPSCs from inflamed pulp (DPSCs-IP) multiple colony (MC), sample #1, 17-year-old male tooth #19. **(C–F)** DPSCs-IP, sample #2, 19-year-old male tooth #30 ((**C**) MC, (**D**) SC-4a, (**E**) SC-5a and (**F**) SC-6b). **(G & H)** DPSCs-IP sample #3, 15-year-old male tooth #30 ((**G**) MC and (**H**) SC-6a). **(I)** Analysis of the amount of dentin-like mineralized tissues formed by DPSCs between different samples. The labels of the x-axis (**A–H**) correspond to the (**A–H**) images.

Arrows: mineralized tissue; arrowheads: odontoblast-like cells.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; error bars, standard error of the mean; $n = 7$.

D: Dentin-like; DP: Dental pulp-like; HA: Hydroxyapatite; Od: Odontoblast-like.

Table 1

Lineage-specific differentiation-inducing media.

Lineage	Medium	Serum	Supplementation
Osteo/dentinogenic	DMEM	10% FBS	10 nM dexamethasone, 10 mM β -glycerophosphate, 50 μ g/ml ascorbate phosphate and 10 nM 1,25 dihydroxyvitamin D3
Adipogenic	DMEM	10% FBS	1 μ M dexamethasone, 1 μ g/ml insulin and 0.5 mM 3-isobutyl-1-methylxanthine
Neurogenic	Neurobasal A	Serum free	B27 supplement, 20 ng/ml EGF and 40 ng/ml FGF

Medium/serum and supplement were changed every 3 days.

DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum.

Data taken from [14,24,41].

Table 2

Specific primers for real-time reverse transcription-PCR.

Lineage	Gene	Primer (5' --- 3')	
		Sense Antisense	Product size (bp)
Osteo/dentinogenic	<i>DSPP</i>	TCACAAGGGAGAAGGGAATG TGCCATTGCTGTGATGTTT	182
	<i>BSP</i>	AAAGTGAGAACGGGAACCT GATGCAAAGCCAGAATGGAT	161
	<i>OCN</i>	GGCAGCGAGGTAGTGAAGAG CTGGAGAGGAGCAGAACTGG	230
	<i>ALP</i>	CCACGTCTTCACATTGTTGGT AGACTGCGCCTGGTAGTTGT	196
	<i>CBFA1</i>	TTTGCACTGGGTCATGTGTT TGGCTGCATTGAAAAGACTG	156
Adipogenic	<i>LPL</i>	AGTGGCCAAATAGCACATCC CCGAAAGATCCAGAATTCCA	186
	<i>PPARγ2</i>	TCCATGCTGTTATGGGTGAA TCAAAGGAGTGGGAGTGGTC	193
Neurogenic	<i>βIII tubulin</i>	CAGATGTTTCGATGCCAAGAA GGGATCCACTCCACGAAGTA	181
	<i>Nestin</i>	AACAGCGACGGAGGTCTCTA TTCTCTTGTCCTCCGAGACTT	220
	<i>CNPase</i>	GTGGAGCACAAAAGCCTCTC AAGTTTCCCATGTGGCTGAC	251
	<i>NFM</i>	TGGGAAATGGCTCGTCATTT CACCTGCCTGCTTTAACTT	198
Housekeeping	<i>GAPDH</i>	CAAGGCTGAGAACGGGAAGC AGGGGGCAGAGATGATGACC	194

Table 3

Density of marker expression in pulp.

Marker	Stained loci count [†]		p-value
	<i>Normal pulp</i>	<i>Inflamed pulp</i>	
STRO-1	2.67 ± 1.49 n = 10	3.64 ± 1.60 n = 11	≤0.016
CD90	11.76 ± 3.36 n = 7	18.89 ± 7.93 n = 9	≤0.008
CD105	4.83 ± 3.3 n = 10	9.97 ± 5.6 n = 10	≤0.0001
CD146	7.29 ± 2.90 n = 8	9.33 ± 4.25 n = 8	≤0.027

[†] For stained loci count, three areas were randomly selected and counted at ×400 for every sample section (n), and then nested within the sample section for statistical analysis. ANOVA with a nested design was performed. Data are listed as mean ± standard deviation.

Table 4

Population doubling study[†].

Sample number	DPSCs-NPs		Sample number		DPSCs-IPs	
	Age/gender/tooth#	MC [‡] /SC [§] PD [¶]	Age/gender/tooth#	MC [‡] /SC [§] PD [¶]	Age/gender/tooth#	MC [‡] /SC [§] PD [¶]
1	9/F/#11	MC	70.0	1	17/M/#19	MC
		SC-2a	57.4	2	19/M/#30	MC-1
		SC-2b	26.2		MC-2	40.1
		SC-4a	71.7		SC-4a	19.7
2 (same donor as 1)	9/F/#6	MC	63.1		SC-5a	21.3
		SC-1a	50.2		SC-5b	50.1
		SC-2a	39.7		SC-6a	34.3
		SC-4a	15.7		SC-6b	34.7
3	19/F/#17	MC	55.1	3	15/M/#30	MC
					SC-2a	8.5
					SC-2b	34.6
					SC-3a	40.8
4	16F#1	MC	62.1		SC-6a	33.6
		SC-1a	46.1			
		SC-1b	51.8			
		SC-1c	28.7	4	16M#12	SC-1a
5	16F#20	MC	57.3		SC-1b	18.1
		SC-1a	62		SC-1c	21.1
		SC-1b	52.7	5	15F#31	MC
		SC-1c	67.6		SC-1a	26.4
		SC-1d	65.9		SC-1b	25.3
					SC-1c	25.6
					SC-1d	23.8

Sample number	DPSCs-NPs		Sample number		DPSCs-IPs	
	Age/gender/tooth#	MC [‡] /SC [§]	PD [¶]	Age/gender/tooth#	MC [‡] /SC [§]	PD [¶]
6	21M#16	MC	52.1			
6 (cyto ^{††})	21M#16	MC	43.5	12M#19	MC	35.3
7 (same donor as 6)	21M#17	MC	52.3			
7 (cyto ^{††})	21M#17	MC	47.8	17M#10	MC	39.1

[‡] DPSCs were isolated from each tooth.

[‡] MC: Multiple colony-derived DPSCs from the original pool of cell colonies.

[§] SC: Single colony-derived DPSCs selected from the original pool of colonies. The numbers and small letter of SC are the clone names.

[¶] PDs were derived and calculated as described in the Materials & methods.

^{††} cyto: Cytokine stimulated (TNF- α plus IL-1 β).

DPSC: Dental pulp stem cell; F: Female; IP: Inflamed pulp; M: Male; NP: Normal pulp; PD: Population doubling.