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RESEARCH ARTICLE

Growth and Osteogenic Differentiation of CD117+ Dental Pulp and Periodontal Ligament Cells

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Abstract

BACKGROUND: Dental pulp stem cell (DPSC) and periodontal ligament stem cell (PDLSC) have been suggested as valuable seed cells for bone engineering, suggesting that both stem cells are potential osteogenic sources. Since DPSC and PDLSC seem like to have similar potential in bone formation, we conducted a study to compare morphology, immunophenotype and cell growth of DPSC and PDLSC isolated from the same teeth.

METHODS: Human dental pulps and periodontal ligaments were obtained from freshly extracted partial impacted third molar teeth. Collected samples were digested with type I collagenase. Resulted cell suspension was washed and cultured. For biomarker identification, the cells were fixed and bound with anti-fluorescein isothiocyanate (FITC)-cluster of differentiation (CD)117 antibody. For cell growth quantification, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used. Meanwhile for osteogenic differentiation, the cells were cultured in osteogenic medium for 1-3 weeks, fixed and stained with alizarin red.

RESULTS: Morphology of dental pulps cell (DPC) and periodontal ligament cell (PDLC) in passage 5 was similar. Clear CD117 green fluorescence of DPC and PDLC in passage 5 was observed. Cell growth rate of PDLC was higher than the one of DPC, 0.3858 and 0.3848 respectively. DPC formed bone nodule on the third week culture in osteogenic medium, while PDLC showed bone nodule formation on the second week culture.

CONCLUSION: We suggest that DPC and PDLC are potential seed cells for osteogenic regeneration, since they had cell growth capacity and osteogenic differentiation, particularly PDLC that had faster osteogenic differentiation.

KEYWORDS: dental pulp, periodontal ligament, cell, growth, osteogenic differentiation

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Introduction

Stem cell has been reported useful for cell and tissue regeneration.(1-4) Dental pulp stem cell (DPSC) is able to self-renew and to multi-lineage-differentiate.(5) Under stimulation, DPSC can be differentiated towards various cell types, such as adipocytes, myoblasts, neurons, chondrocytes and osteoblasts.(1,5-7) Therefore DPSC could be a potential source for reconstructive medicine, replacing lost or

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damaged tissues.(5) To find out potential DPSC source, DPSC derived from human third molar and from deciduous incisor were compared.(8) Results show that DPSC from the human third molar has higher proliferation capacity, which was suggested as an appropriate candidate for use in experimental, preclinical and even clinical setups.(8) DPSC has been reported to be useful to regenerate oral tissues, including dental pulp (9) and bone (10).

To date, 5 different human dental stem/progenitor cells have been isolated and characterized: DPSC, stem cell from exfoliated deciduous teeth (SHED), periodontal ligament stem cell (PDLSC), stem cell from apical papilla (SCAP), and dental follicle progenitor cell (DFPC).(11) Among the dental stem cells, DPSC and PDLSC were reported to have osteo-/dentino-genic property.(11) Therefore DPSC has been reported to be valuable as seed cells for bone tissue engineering.(12) Meanwhile, PDLSC was also reported to have greater calcium deposition after 3-week culture.(13)

In an *in vivo* study, dental tissue-derived adult stem cells, including DPSC and PDLSC were autologous transplanted into the apical involvement defect.(14) PDLSCs showed the best regenerating capacity of periodontal ligament, alveolar bone, and cementum as well as peripheral nerve and blood vessel, which were evaluated by conventional immunohistology, 3D micro-CT and clinical index.(14) PDLSC is suggested as a favorable candidate for the clinical application, that can be used for treatment of advanced periodontitis where tooth removal was indicated in the clinical cases.(14) Since DPSC and PDLSC were both reported as potential stem cells in bone formation, we conducted a study to compare DPSC and PDLSC derived from the same teeth.

Methods

Sample Collection

Human dental pulps and periodontal ligaments were obtained from extracted partial impacted third molars. Periodontal ligaments and pulps were transported to the laboratory in microcentrifuge tubes containing complete medium (Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS) (Gibco) and antibiotic-antimycotic containing 200 units/mL penicillin, 200 µg/mL streptomycin and 0.5 µg/mL amphotericin (Gibco)). Experimental procedures were approved by the Ethics Committee of Faculty of Dentistry Trisakti University, Indonesia (#167/KE/FKG/11/2014).

Primary Dental Pulp Cell (DPC) and Periodontal ligament Cell (PDLC) Cultures

DPC and PDLC cultures were established by enzyme digestion, as previously we conducted for lipoaspirates.(15) Dental pulps and periodontal ligaments were treated with 3 mg/mL type I collagenase (Gibco) and 4 mg/mL dispase (Gibco) for 120 minutes at 37ºC. After enzymatic digestion, the cell suspension was washed by centrifugation for 5 minutes in complete medium and seeded into 3.5-cm culture dish. The culture was maintained in complete medium in a humidified incubator with 5% CO_2 atmosphere at 37ºC.

Immunofluorescence

DPC and PDLC were washed twice with PBS and fixed in ice cold 70% ethanol for 60 minutes at room temperature. The fixative was carefully removed, and the cells were gently washed three times with distilled water and stained with FITC-conjugated mouse monoclonal anti-CD117 antibody (Biobyt Ltd., Cambridge, UK) for 20 minutes. Then the plates were washed three times with distilled water. To have better cell determination, 4',6-diamidino-2-phenylindole (DAPI) (Wako, Osaka, Japan) staining was performed. Cell observation and documentation was conducted under an inverted fluorescence microscope (Axio Observer, Carl Zeiss, Jena, Germany).

Cell Growth Assay

Cell growth was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA). The MTT assay provides a quantitative measurement of viable cells by determining the amount of formazan crystals-produced by metabolically active cells. Briefly, DPC and PDLC in different numbers were seeded into a 96-well plate. After 1, 2 or 3 days of incubation, 10 µL of 5 mg/mL MTT in phosphate buffer saline (PBS) was added to each well. The plate was then incubated in a humidified atmosphere of 5% CO_2 at 37ºC for 4 hours. Medium was discarded and formazan crystals were dissolved in 1.0 mL of 0.1 N HCl. Absorbance of DPC and PDLC were measured at 570 nm by a microplate reader. In addition, DPC and PDLC numbers were counted with a hemacytometer and used for interpolating the absorbance of MTT assay results. Doubling time and growth rate were measured using Cell Calculator++ at *www.doubling-time.com.*

Osteogenic Functional Test

Osteogenic medium consisting of 50 µg/mL L-ascorbic acid (Sigma Aldrich), 100 nM dexamethasone (Sigma-

Aldrich), 10 mM β-glycerophosphate (Sigma-Aldrich) was used to culture DPC and PDLC. On the seventh, fourteenth or twenty-first day, the medium was carefully aspirated from each plate, washed twice with PBS, fixed in freshly prepared 4% paraformaldehide (Wako, Osaka Japan) in PBS for 2 minutes, followed by glycerol (Biorad, Richmond, CA, USA) for 5 minutes at room temperature. Then fixative was carefully removed, and the cells were gently washed three times with distilled water and stained with 2% alizarin red (Sigma) for 20 minutes. After alizarin red solution was removed and the plates were washed three times with distilled water. Documentation was performed under an inverted light microscope.

Statistical Analysis

Data were statistical analyzed using IBM SPSS Statistics for Macintosh version 21 (SPSS IBM, Armonk, NY, USA). Normality tests were conducted using Shapiro Wilk. Comparison of 3 independent groups was conducted using Kruskal Wallis. Comparison of 2 independent groups was conducted using Independent Sample T or Mann Whitney. Statistically significance is expressed as $p<0.05$.

Results

Human dental pulps and periodontal ligaments were obtained from 7 freshly extracted partial impacted third molars. Periodontal ligaments were immediately collected after the extraction, followed by rapid teeth sectioning and pulps collection.

Morphology of DPC and PDLC

Morphology of DPC and PDLC in passage 5, are shown in Figure 1. The morphology of both DPC and PDLC are similar and fibroblast-like.

CD117 expression of DPC and PDLC

CD117 expression of DPC and PDLC in passage 5, are shown in Figure 2 (A-E). Clear CD117 green fluorescence was documented for both DPC and PDLC. CD117 green fluorescence was observed at the cell membranes, while nuclear DAPI staining was also observed to mark each detected cell.

Growth of DPC and PDLC

Growth of DPC and PDLC in passage 5, are shown in Fig. 3A. Average number of viable PDLC was significantly higher than number of viable DPC (Kruskal Wallis, p=0.004). Both DPC and PDLC grew significantly in a time dependent manner (Kruskal Wallis, p=0.000). Number of DPC on the second day culture was significantly higher than the first day culture (Independent Sample T, p=0.04), while number of PDLC on the second day culture was also significantly higher than the first day culture (Mann Whitney, p=0.008). Number of DPC on the third day culture was significantly higher that the second day culture (Mann Whitney, p=0.001), while number of PDLC on the second day culture was also significantly higher that the first day culture (Mann Whitney, p=0.000). Both DPC and PDLC showed similar doubling time and growth rate (Figure 3B and C).

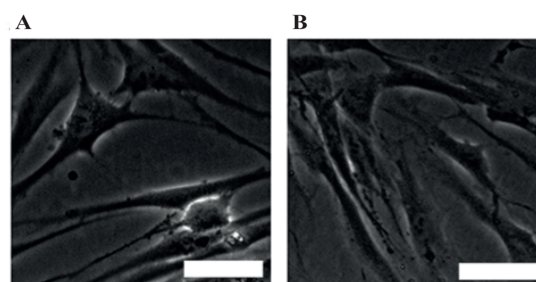


Figure 1. Morphology of cells derived from dental pulps and periodontal membranes. Isolated cells derived from dental pulps (**A**) and periodontal membranes (**B**) were cultured according to Materials and Methods. Cells in passage 5 were documented under an inverted light microscope. White bar: 10 μm.

Osteogenic differentiation of DPC and PDLC

As shown in Figure 4, representative images of alizarin red staining of bone nodule formation by DPC and PDLC. DPC could form bone nodule on the third week culture in osteogenic medium. Meanwhile PDLC showed bone nodule formation on the second week culture.

Discussion

Our results showed that both DPC and PDLC had similar growing capacity. In accordance to previous report (16), the growth rate of DPC was about 0.3858, and the doubling time was 1.8 day (43.2 hours). Meanwhile for PDLC, based on our current research results, similar growth rate and doubling time were found. These results were also in accordance to another previous report.(17) These results suggested that both DPC and PDLC could be potentially be enriched when certain numbers of cells are needed. However, DPC with shorter doubling time (20.79 hours) was reported (8), therefore current cell culture should be optimized.

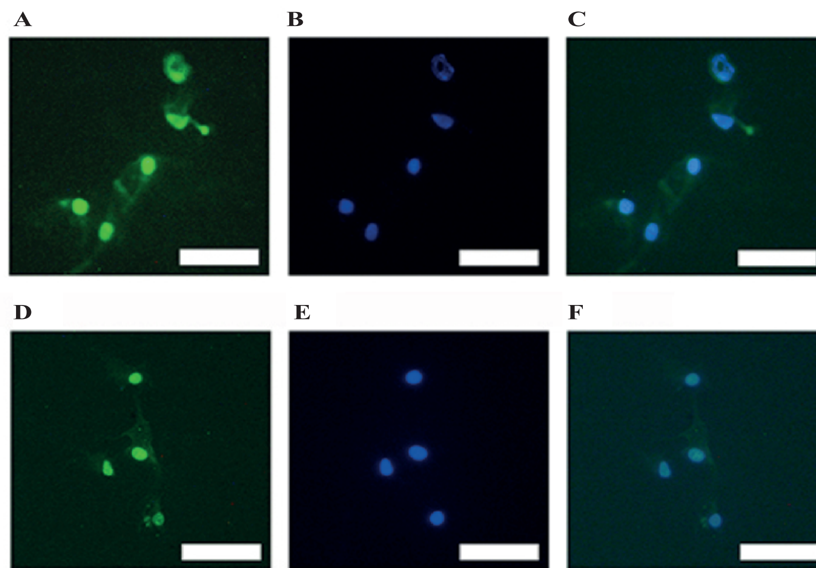
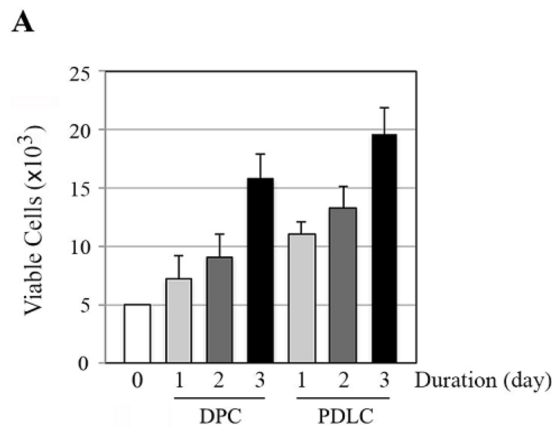
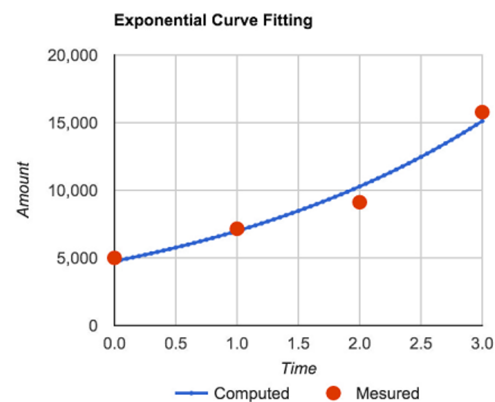


Figure 2. CD117 expression of cells derived from dental pulps and periodontal membranes. Cells in passage 5 derived from dental pulps (**A**, **B** & **C**) and periodontal membranes (**D**, **E** & **F**) were fixed and stained with anti-CD117 antibody and DAPI according to Methods. Resulted fluorescence was captured under an inverted fluorescence microscope. **A** & **D**: CD117 expression; **B** & **D**: DAPI expression; **C**: Merge of **A** & **B**; **F**: Merge of **D** & **E**; White bar: 30 µm.



B

Doubling Time = 1.8=ln(2)/0.3858
Growth Rate = 0.3858
Growth Rate = number of doublings that occur per unit of time
Equation : amount=4752.7917*$e^{0.3858*time}$
At t=0, calculated cell concentration = 4752.7917



C

Doubling Time = 1.8=ln(2)/0.3848
Growth Rate = 0.3848
Growth Rate = number of doublings that occur per unit of time
Equation : amount=6316.3278*$e^{0.3848*time}$
At t=0, calculated cell concentration = 6316.3278

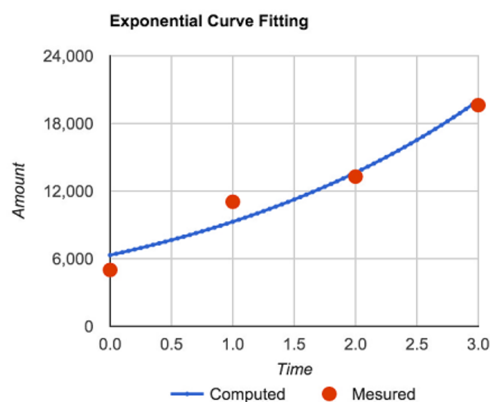


Figure 3. Growth of DPC and PDLC. DPC and PDLC in passage 5 were cultured for 1, 2 and 3 days. **A**: Viable cells were counted with MTT assay according to Materials and Methods. **B**: Doubling time and growth rate of DPC. **C**: Doubling time and growth rate of PDLC. This experiment was performed in duplicate and performed five times.

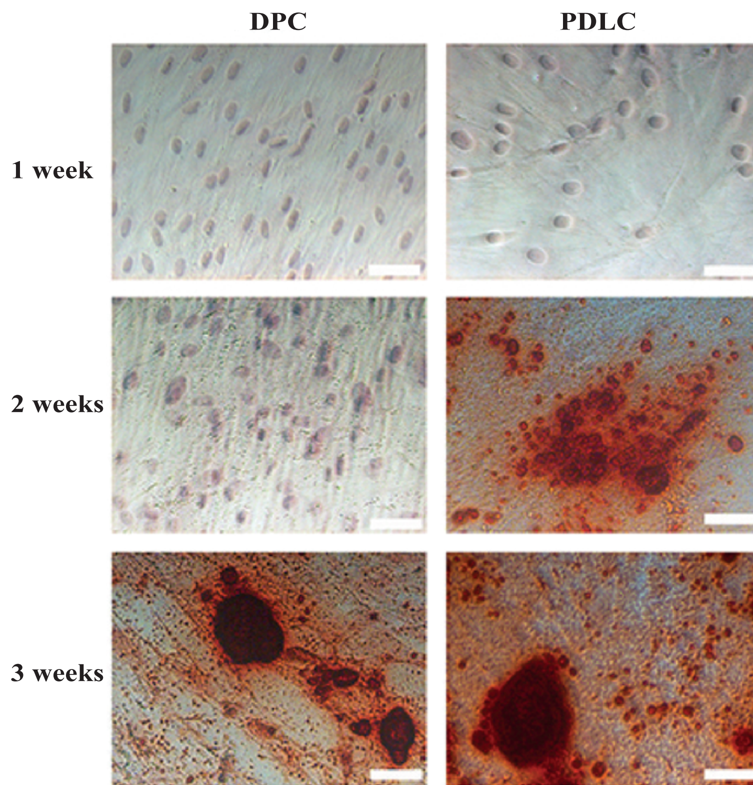


Figure 4. Osteogenic expression of DPC and PDLC. DPC and PDLC in passage 5, were cultured for 1, 2 or 3 weeks. Then the cells were fixed and stained with alizarin red according to Materials and Methods. Documentation was performed under an inverted light microscope. White bar: 30 µm.

CD117 has been reported to be expressed in adult / neonatal DPC (18,19) and PDLC (20). When stem cell factor (SCF) binds to CD117, cell proliferation, cell migration, angiogenesis and tissue remodeling can be induced.(21,22) There are some underlying mechanisms of SCF to activate those activities, amongst them is the phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK signaling pathways.(21) Our current study showed clear expression of CD117 on both DPC and PDLC, suggesting that isolated cells might have multipotent capacities.

DPSC was reported to exhibit a higher osteogenic potential compared with bone marrow stem cell both *in vitro* and *in vivo*, making it a potential cell source for future bone tissue engineering.(23) Osteogenic differentiation of DPSC can be observed with alizarin red stain after 3 weeks of induction.(24) Our current results also showed that DPC osteogenic differentiation was observed after 3-week period. Meanwhile, osteogenic differentiation observed with alizarin red stain of PDLC was observed after 2 weeks, faster than DPC. These results suggested that PDLC could be a better cell source in terms for osteogenesis.

Some other types of oral tissue derived stem cell including SHED, SCAP and DFPC (11), however were not investigated in this study, therefore possibility of better osteogenic stem cell source should be further pursued. Furthermore, since this study was suggested to support the regenerative tissue in oral area, therefore not only osteogenic regeneration, other tissue regeneration such as soft tissue, pulpal-, neuro-regenenération should be also considered.

Conclusion

Hence, taken together we suggest that DPC and PDLC are potential seed cells for osteogenic regeneration, since they had cell growth capacity and osteogenic differentiation, particularly PDLC that had faster osteogenic differentiation, therefore should be investigated further for their potential for other tissue regeneration.

Acknowledgments

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