J Clin Periodontol 2011; 38: 721–731 doi: 10.1111/j.1600-051X.2011.01716.x

Journal of Clinical Periodontology

Isolation and characterization of human periodontal ligament (PDL) stem cells (PDLSCs) from the inflamed PDL tissue: in vitro and in vivo evaluations

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Park J-C, Kim J-M, Jung I-H, Kim JC, Choi S-H, Cho K-S, Kim C-S. Isolation and characterization of human periodontal ligament (PDL) stem cells (PDLSCs) from the inflamed PDL tissue: in vitro and in vivo evaluations. J Clin Periodontol 2011; 38: 721–731. doi: 10.1111/j.1600-051X.2011.01716.x.

Abstract

Objectives: Mesenchymal stem cells (MSC) could be isolated from healthy periodontal ligaments (PDL). The aims of this study were to isolate and characterize human PDL stem cells (hPDLSCs) from inflamed PDL tissue, and to evaluate their regenerative potential.

Materials and Methods: Inflamed hPDLSCs (ihPDLSCs) were isolated from the inflamed PDL tissue obtained from intra-bony defects during flap surgery, and characterized by immunohistochemical staining, colony-forming unit assay, fluorescence-activated cell sorting, and mRNA expression in comparison with healthy hPDLSCs obtained from extracted teeth for orthodontic purpose. The proliferative potential and migratory potential was evaluated, and compared with healthy hPDLSCs. Regenerative potential was assessed by an in vivo ectopic transplantation model.

Results: ihPDLSCs were successfully isolated and characterized as MSCs. Both ihPDLSCs and hPDLSCs were successfully differentiated under osteogenic/cementogenic and adipogenic microenvironment. The proliferative potential did not differ between healthy hPDLSCs and ihPDLSCs, while the migratory capacity was significantly increased in ihPDLSCs ($p < 0.05$). Both groups exhibited new cementum-like tissue and related PDL fibre regeneration in an in vivo transplantation model.

Conclusion: ihPDLSCs could be successfully isolated from inflamed PDL tissue, and they retained the regenerative potential for cementum and related PDL tissues.

Key words: inflammation; periodontal ligament; periodontal ligament stem cells; regeneration; tissue engineering

Accepted for publication 7 February 2011

The concept that the periodontal ligament (PDL) contains a mixed population of cells that can synthesize various tissues has been suggested by several authors (Roberts & Jee 1974, Melcher 1976). These cells appear around blood vessels in the PDL, and have been identified as progenitor cells by McCulloch (1985). However, these paravascular progenitor cells in the PDL space cannot be designated as stem cells, because the basic characteristics of stem cells (i.e., the abilities to self-renew, generate large numbers of progeny, and differentiate into multiple mature cell types) (Fortier 2005) have not been demonstrated.

Multipotent human PDL stem cells (hPDLSCs) were eventually isolated by single-colony selection and magnetic-activated cell sorting, and characterized according to their expression of the mesenchymal stem cell (MSC) markers STRO-1 and CD146/MUC18 (Seo et al. 2004). The hPDLSCs isolated from the PDL tissue of impacted wisdom teeth shared the characteristics of other

Conflict of interest and source of funding statement

The authors declare that there are no conflicts of interest in this study.
This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 2009-0078884).

postnatal human MSCs, including formation of colony-forming units (CFU) and possession of osteogenic, adipogenic, chondrogenic, and neurogenic differentiation capabilities. These hPDLSCs could also form a thin layer of cementum-like tissue on the surface of hydroxyapatite/tricalcium phosphate (HA/TCP) particles along with condensed collagen fibres resembling Sharpey's fibres, when transplanted with HA/TCP as a cell carrier into an in vivo ectopic transplantation model in immunodeficient mice.

There are currently various dental-origin MSCs other than hPDLSCs, including dental pulp stem cells (DPSCs) (Gronthos et al. 2002), stem cells from the apical papilla (SCAP) (Sonoyama et al. 2006, 2008), and stem cells from human exfoliated deciduous teeth (SHED) (Miura et al. 2003, Kerkis et al. 2006). Until now, impacted third molars or teeth that have been extracted for orthodontic purposes have been the most favored sources for DPSCs and hPDLSCs, but these are not always available when needed. Furthermore, SCAP and SHED are not available in adults. Therefore, alternative, readily accessible sources are continuously being sought.

In the search for alternative sources of MSCs, inflamed dental tissues have been assessed for their potency. It was recently demonstrated that stem/progenitor cells could be successfully isolated from inflamed adult human dental pulp (Alongi et al. 2010), and the presence of putative stem cells in periodontitis-affected PDL tissue was also demonstrated (Chen et al. 2006). Furthermore, stem cells isolated from periodontitis-affected PDL tissue were even shown to differentiate into highly proliferative neural precursors in vitro (Widera et al. 2007). However, few if any studies have investigated the regenerative potential of MSCs obtained from inflamed PDL tissue.

The aims of this study were to isolate and characterize human PDL stem cells from healthy or inflamed human PDLs (hPDLSCs and ihPDLSCs, respectively), and to evaluate their regenerative potentials. We demonstrated that it was possible to isolate hPDLSCs from the inflamed tissue of human periodontal defects during periodontal flap surgery, and that these cells (ihPDLSCs) retained the regenerative potentials.

Materials and Methods

Isolation of hPDLSCs

hPDLSCs were isolated and cultured according to previously reported protocols with slight modification (Seo et al. 2004). Briefly, human premolars extracted from six systemically healthy adults (16–29 years of age) at the Department of General Dentistry, Dental College, Yonsei University, Seoul, Korea for orthodontic purposes were used under approved guidelines set by the Institutional Review Board, College of Dentistry, Yonsei University. PDL tissues were separated from the root surface using a scalpel and were minced into the smallest size possible. The minced PDL tissues were digested five times at 30-min. intervals in α-minimum essential medium (α-MEM; Gibco, Grand Island, NY, USA) containing 3 mg/ml collagenase type I (WAKO, Tokyo, Japan) and 4 mg/ml dispase (Gibco) at 37°C. Single-cell suspensions were obtained by passing the cell-containing medium through a strainer with a pore size of 70 μm (BD Falcon Labware, BD Labware, Franklin lakes, NJ, USA). The resulting cells (5×10^5) were seeded onto T75 cell culture dishes containing a growth medium of α-MEM supplemented with 15% foetal bovine serum (FBS; Gibco), 100 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco), and incubated at 37°C in 5% CO_2. Single-cell colonies were observed and passage 0 (P0) cells were cultured. Cells at passages P3–P5 were used for the study.

Isolation of ihPDLSCs from the inflammatory granulation tissue of periodontal defects

Six patients between 42 and 57 years of age with moderate or severe chronic peridontitis were selected as subjects at the Department of Periodontology, College of Dentistry, Yonsei University. The inflammatory granulation tissue was isolated from the periodontal defect of these systematically healthy patients. Surgical sites had a periodontal probing pocket depth of >6 mm with bleeding on probing and radiographic evidence of intra-bony pockets larger than 3 mm at the remaining two or three walls. All of the surgical procedures were performed for the purposes of treatment. Patients received a professional prophylactic treatment including scaling and root planing before periodontal flap surgery. The study was reviewed and approved by the Institutional Review Board of the College of Dentistry, Yonsei University, and all patients gave their informed consent to participate.

Simplified papilla-preservation flaps were raised after administering local anaesthesia (Cortellini et al. 1999). Unattached necrotic granulation tissue was removed roughly using an ultrasonic scaler, and then inflammatory granulation tissue attached at the base of the intra-bony defect was removed for experimental usage. The defects and surgical area were cleaned thoroughly by hand instruments and with the aid of ultrasonic devices. After the planned treatment, the flaps were repositioned and closed with interrupted sutures.

Inflammatory granulation tissue samples larger than 3 × 3 mm were collected into the aforementioned growth medium. Immediately after collection, the tissues were subjected to the cell-isolation procedure using enzymatic digestion, as described above. Cells isolated from the inflammatory granulation tissue were named ihPDLSCs. Part of the granulation tissue was used for histological and immunohistochemical evaluation, whereby it was embedded and frozen in OCT compound (Tissue-Tek 4583, Sakura Finetek, Tokyo, Japan). Serial frozen sections were cut at 7 μm on a cryostat (CM3050S, Leica, Wetzlar, Germany) and stained with haematoxylin and eosin (H–E). The histological analyses were performed with the aid of both light and fluorescence microscopy (BX50, Olympus Optical, Tokyo, Japan).

For immunohistochemical evaluation, sections were blocked with blocking solution (Human Mesenchymal Stem cell Characterization Kit, Cat. No. SCR067, Millipore, Billerica, MA, USA) and then incubated with primary antibody (mouse anti-STRO-1, Human Mesenchymal Stem Cell Characterization Kit) overnight at 4°C. After washing with phosphate-buffered saline (PBS), sections were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 2 h at room temperature. After washing with PBS, sections were incubated again with primary antibody (rabbit anti-CD146, clone EPR3208, Millipore) and then with Texas red-conjugated secondary antibody. Cells were counterstained with the nuclear dye 4′,6-diamidino-2-

phenylindole (DAPI, 1:100; Vector Laboratories, Burlingame, CA, USA), and images were obtained with the confocal laser scanning microscopy (LSM 700 Laser Scanning Microscope, Carl Zeiss Inc., Thornwood, NY, USA).

Characterization of isolated ihPDLSCs as MSCs: CFU assay

A CFU assay was performed to determine the presence of putative MSCs in the granulation tissue taken from periodontal defects. Cells were plated at 1×10^2 cells/ml onto 100-mm culture dishes with growth media, and 14 days thereafter the dishes were fixed with 4% formaldehyde and then stained with crystal violet (Sigma-Aldrich). Cells were observed under light microscope (BX50, Olympus Optical) after 4, 9 and 14 days.

Characterization of isolated ihPDLSCs as MSCs: immunocytochemical analysis

Cells (P3) were subcultured into two chamber slides. The cells were fixed in 4% paraformaldehyde for 30–45 min. and then blocked with blocking solution (Human Mesenchymal Stem Cell Characterization Kit). They were then incubated with primary antibodies (anti-STRO-1, anti-CD146, anti-CD90, and anti-CD44, Human Mesenchymal Stem Cell Characterization Kit) overnight at 4°C. After washing with PBS, cells were incubated with Cy3-conjugated secondary antibody for 45 min. at room temperature. Cells were counterstained with the nuclear dye DAPI (1:100, Vector Laboratories). Images were analysed using light and fluorescence microscopy (BX50, Olympus Optical).

Characterization of isolated ihPDLSCs as MSCs: cell-surface-marker characterization

Cell-surface-marker characterization of ihPDLSCs was performed using flow cytometry analysis, the procedure for which is described elsewhere (Shi & Gronthos 2003) in comparison with hPDLSCs. ihPDLSCs and hPDLSCs in T75 flasks were treated with trypsin–EDTA. After the cells were harvested and transferred to a 1.7 ml tube (Oxygen, Union City, CA, USA), they were fixed by adding 4% paraformaldehyde for 15 min. The cells were then incubated with 3% bovine serum albumin and then with primary antibodies raised against STRO-1, CD146, CD90, CD44, CD19, or CD14 for 1 h. The cells were then washed with wash buffer, and the secondary antibody (to which FITC was attached) was added for 45 min. at room temperature. The cells were then washed three times and observed with a flow cytometer (FACSCalibur, BD Biosciences, Franklin lakes, NJ, USA).

Induction of osteogenic and adipogenic differentiations

Cells were cultured and osteogenic and adipogenic differentiations were induced. Cells (P4) were seeded into six-well plates at 1×10^5 cells/well, and cultured until they reached subconfluence. The culture medium for osteogenic differentiation comprised α-MEM (Gibco) containing 15% FBS (Gibco), 2 mM L-glutamine (Gibco), 100 μM L-ascorbic acid 2-phosphate, 10^{-8} M dexamethasone (Sigma-Aldrich), 2 mM β-glycerophosphate (Sigma-Aldrich), 55 μM 2-mercaptoethanol (Amresco, Solon, OH, USA), 1.8 mM KH_2PO_4 (Sigma-Aldrich), 100 U/ml penicillin (Gibco), and 100 μg/ml streptomycin (Gibco), and was refreshed at 3-day intervals. The culture medium for adipogenic differentiation comprised α-MEM containing 15% FBS, 2 mM L-glutamine, 100 μM L-ascorbic acid 2-phosphate, 500 μM isobutyl-methylxanthine (Sigma-Aldrich), 60 μM indomethacin (Sigma-Aldrich), 5 μM hydrocortisone (Sigma-Aldrich), 10 μM insulin (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin. After 2 weeks (adipogenic differentiation) or 4 weeks (osteogenic differentiation) of induction, the cells were stained with oil red O stain or alizarin red, respectively, and the total area of mineralized nodule formation and lipid vacuoles were measured using an automated image-analysis system (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA).

Total RNA extraction and reverse transcriptase (RT)-polymerase chain reaction (PCR)

Total RNA was isolated from in vitro culture specimens using TRIzol reagent (Invitrogen, Carlsberg, CA, USA). The RT-PCR was performed with 1 μg of RNA using a commercial kit (Cat. No. 25081, Maxime RT PreMix, Oligo dT primer, iNtRon Biotechnology, Daejeon, Korea). The primers used are listed in Table 1. PCR products were visualized by electrophoresis on agarose gels stained with ethidium bromide and analysed with the aid of a gel-documentation system (Gel Doc XR, Bio-Rad, Hercules, CA, USA).

Assessment of proliferation and migratory potentials

Cell proliferation was assessed using the MTT assay, the bromodeoxyuridine (BrdU; Roche, Mannheim, Germany)-labelling efficacy assay, and cell-cycle analysis. Cells (1×10^3) were plated on 48-well plates and cultured, and the MTT assay from days 1 to 12 was performed. After incubation with 10% MTT solution (5 mg/ml; Amresco) and culture media at 36°C, 5% CO_2 for 4 h, the supernatant was discarded, and dimethylsulphoxide (Amresco) was added to each well. The absorbance was measured at 540 nm using a microplate spectrophotometer [Benchmark Plus enzyme-linked immunosorbent assay (ELISA) reader, Bio-Rad].

The DNA synthesis of hPDLSCs and ihPDLSCs was assessed by measuring BrdU incorporation using a cell-proliferation ELISA kit according to the manufacturer's instructions and the methods of Zheng et al. (2009). Cells (5×10^4) were plated in 96-well plates and cultured for 24 h, after which BrdU labelling solution (10 μM) was added to each well and the cells were re-incubated for 2 h at room temperature to allow BrdU to incorporate into the newly synthesized DNA of the proliferating cells. After discarding the remnant labelling medium, the cells were fixed with FixDenat solution for 30 min., and incubated again with anti-BrdU-PDO working solution for 90 min. at room temperature. Cells were rinsed with PBS three times, and then the substrate solution was added. After allowing time for sufficient colour development, the absorbance of the incorporated BrdU was measured using an ELISA microplate reader (Bio-Rad) at 540 nm.

Cell-cycle analysis was performed. A 3-ml aliquot of cold absolute ethanol was added drop by drop to the cell suspension of hPDLSCs and ihPDLSCs while vortexing to a final ethanol concentration of 70%, and then the suspension was stored for at least 1 h at 4°C. Finally, the cells were washed twice with PBS (centrifuged at 500 g for 5 min.), incubated with 1 ml of propidium iodide staining solution (50 μg/ml; Sigma-Aldrich), and mixed well. A 50-μl aliquot of RNase A solution (5 μg/ml; Worthington Biochemicals, Freehold,

Table 1. Primer sequences and condition for reverse transcription-polymerase chain reaction

Gene	Primer sequence		Annealing temperature (°C)	GenBank no.	Product size (bp)
	forward (5′–3′)	reverse (3′–5′)			
Human ALP	TGG AGC TTC AGA AGC TCA ACA CCA	ATC TCG TTG TCT GAG TAC CAG TC	58	93589-007, 93589-008	500
Human Runx2	CAC TGG CGC TGC AAC AAG A	CAT TCC GGA GCT CAG CAG AAT AA	60	NM 001015051.3; NM 001024630.3; NM 004348.3	127
Human OCN	CAA AGG TGC AGC CTT TGT GTC	TCA CAG TCC GGA TTG AGC TCA	60	NM 199173.3	150
Human PPARγ2	CAG TGG GGA TGC TCA TAA	CTT TTG GCA TAC TCT GTG AT	55	93589-001, 93589-002	400
Human aP2	AAC CTT AGA TGG GGG TGT CCT G	TCG TGG AAG TGA CGC CTT TC	58	99226-003, 99226-004	125
Human SCX	CTG GCC TCC AGC TAC ATC TC	CTT TCT CTG GTT GCT GAG GC	58	0052797-001, 0052797-002	209
Human Periostin	CAC AAC CTG GAG ACT GGA C	TGT CTG CTG GAT AGA GGA G	24	NM 015784	322
Human collagen XII	CGG ACA GAG CCT TAC GTG CC	CTG CCC GGG TCC GTG G	28	NM 080645.2	180
β-actin	GGC GGA CTA TGA CTT AGT TG	AAA CAA CAA TGT GCA ATC AA	53	NM 001101.3	238

ALP, alkaline phosphatase; OCN, osteocalcin; PPARγ2, peroxisome proliferator-activated receptorγ2; aP2, adipocyte protein 2; SCX, scleraxis.

NJ, USA) was added, and the mixture was incubated at 4°C for at least 30 min. The cells were then subjected to fluorescence-activated cell sorting (FACS-Caliber, BD Biosciences). One million cells were counted per sample, and the fractions of cells in the G1, S, and G2 phases of the cell cycle were analysed. The multi-cycle mode Modfit LT for Macintosh (version 2.0, BD Biosciences) was used to calculate the fraction of cells in the G0/G1 (represented by the first peak on the histogram), S (between the first and second peaks), and G2-M (the second peak) phases.

Migration activity was analysed by seeding hPDLSCs and ihPDLSCs (1×10^5) into 6-cm-diameter dishes (BD Falcon Labware) and cultured to confluence. Monolayer cells were scratched using blades to make a cell-free area and washed with PBS to eliminate floating cells. Cells were treated with mitomycin C (Acros Organics, Fair lawn, NJ, USA) for 2 h. Cells were observed under a light microscope after 24, 48, and 72 h, and the distance the cells had migrated was measured.

Transplantation of hPDLSCs and ihPDLSCs into the ectopic transplantation model

The cell carrier used for ectopic transplantation was 80 mg of HA/TCP ceramic powder (Biomatlante, Vigneux, France). ihPDLSCs and hPDLSCs (6×10^6 cells/carrier) were precultured for 1.5 h mixed with particles before transplantation at 37°C in 5% CO_2. The carriers loaded with cells were transplanted subcutaneously into the dorsal region of immunodeficient mice. Animal selection, management, and surgical procedures followed a protocol approved by the Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea. The animals were subjected to one of the following three experimental conditions: ihPDLSCs seeded onto the macroporous biphasic calcium phosphate (MBCP) carrier (ihPDLSC group, $n = 6$), hPDLSC seeded on the MBCP carrier (hPDLSC group, $n = 6$) or untreated MBCP carrier (control group, $n = 3$). The animals were allowed to heal for 8 weeks and were then sacrificed for analysis. Block sections were fixed in 4% formalin. The specimens were decalcified with 5% EDTA (pH 8.0) and 4% sucrose, dehydrated in a series of ethanols, and embedded in paraffin. The most central sections of 5 μm thickness were cut at approximately 50 μm intervals, and were stained with H–E. Histological analyses were performed using light and polarized microscopy (BX50, Olympus Optical). The formation and organization of the mineralized and related tissues were observed following Picrosirius staining with polarized light microscopy. For histometric analysis of cementum-like tissue formation, computer-assisted histometric measurements were acquired with an automated image-analysis system (Image-Pro Plus). The outline of newly formed cementum was manually drawn on a captured image of the most central section slide, and the area within the outline was automatically calculated.

For immunohistochemical analysis, deparaffinized sections were immersed in 0.3% hydrogen peroxide to block the endogenous peroxidase activity, and then incubated with primary antibody [mouse monoclonal antibodies against human-specific mitochondria (hMito; Abcam, Cambridge, UK)] diluted in PBS (1:200–1:500). Sections were subsequently counterstained with H–E. Stained sections were analysed under a light microscope and photographed.

Statistical analysis

Unpaired *t*-testing was used to analyse the differences between two groups. For multiple analysis, ANOVA test followed by Scheffé's comparison was used. The level of statistical significance was set at $p < 0.05$. All in vitro experiments were performed in triplicate. All data are presented as mean ± SD values.

Results

Isolation and characterization of ihPDLSCs

The inflammatory nature of the granulation tissue in the periodontal tissue was confirmed by histological evaluation

before the isolation and characterization of the putative stem cells obtained therefrom (Fig. 1). Light microscopy revealed that the granulation tissue obtained from periodontal defects exhibited a mixed pattern of inflammation, which was clearly distinguishable in lower magnification as well as higher magnification, from areas with relatively little inflammation to areas with highly inflamed tissue that was infiltrated with numerous inflammatory leucocytes. Overall, the histology appeared loose, with sparse alignment of connective tissues. Single fibroblast-like cells were frequently observed among the relatively healthy connective tissue fibres. Strongly STRO-1- and CD146-positive (+) cells, which are known to be expressed by PDL stem cells (Seo et al. 2004), were clearly observed following immunohistochemical double staining, suggesting that the putative stem cells resided in the isolated inflammatory granulation tissue.

Putative stem cells were isolated from the obtained granulation tissue as described previously (Seo et al. 2004); their characteristics as MSCs were evaluated in several ways. First, the ability to form CFU was demonstrated (Fig. 2a). Single colonies of ihPDLSCs were formed 7–14 days after the cells were plated at low density. In general, the cells presented a thin and long fibroblastic spindle morphology, and manifested as a heterogeneous population between the subclonal colonies. Second, specific cell-surface markers were selected to show that isolated and ex vivo expanded cells contained the MSC population. ihPDLSCs were stained positive for several primary antibodies including STRO-1, CD146, CD90, and CD44 (Fig. 2b), and flow cytometric analysis confirmed the presence of STRO-1^+, $CD146^+$, $CD90^+$, $CD44^+$, CD19-negative (−), and $CD14^-$ cells in the population (Fig. 2c). Also, the cell surface markers were compared with those of hPDLSCs, and the overall expression patterns were similar between two groups (Fig. 2d).

In addition to their colony-forming ability and the expression of specific antigens on the cell surface, the capacity for differentiation into different mesenchymal tissues is one of the key properties of MSCs. The differentiation potential of ihPDLSCs was evaluated by culturing them in osteogenic and adipogenic media (Fig. 3a–d). After 4 weeks of osteogenic induction, ihPDLSCs exhibited osteogenic potential, as determined by the presence of alizarin-red^+ mineral deposits (indicating calcium accumulation) in vitro in comparison with hPDLSCs. The total area of mineralized nodules were significantly decreased in ihPDLSCs than hPDLSCs (Fig. 3b). Furthermore, ihPDLSCs were capable of undergoing adipogenic differentiation, developing into oil-red-O^+ cells after 2 weeks of induction. The total area of lipid vacuoles were similar between two groups without statistical significance (Fig. 3d). To confirm and compare the differentiation potential of ihPDLSCs and hPDLSCs at the mRNA level, RT-PCR analysis was performed for osteogenic and adipogenic gene markers. Both of cultured ihPDLSCs and hPDLSCs highly expressed a set of osteogenic markers, including alkaline phosphatase, Runx2, and osteocalcin (Fig. 3e), and two adipocyte-related markers: peroxisome proliferator-activated receptor-γ2 and adipocyte protein 2 (Fig. 3f). More importantly, PDL-related mRNAs (Fujii et al. 2008) of scleraxis (Seo et al. 2004), periostin (Horiuchi et al. 1999) and collagen XII (Karimbux et al. 1992) were highly expressed in both groups, suggest-

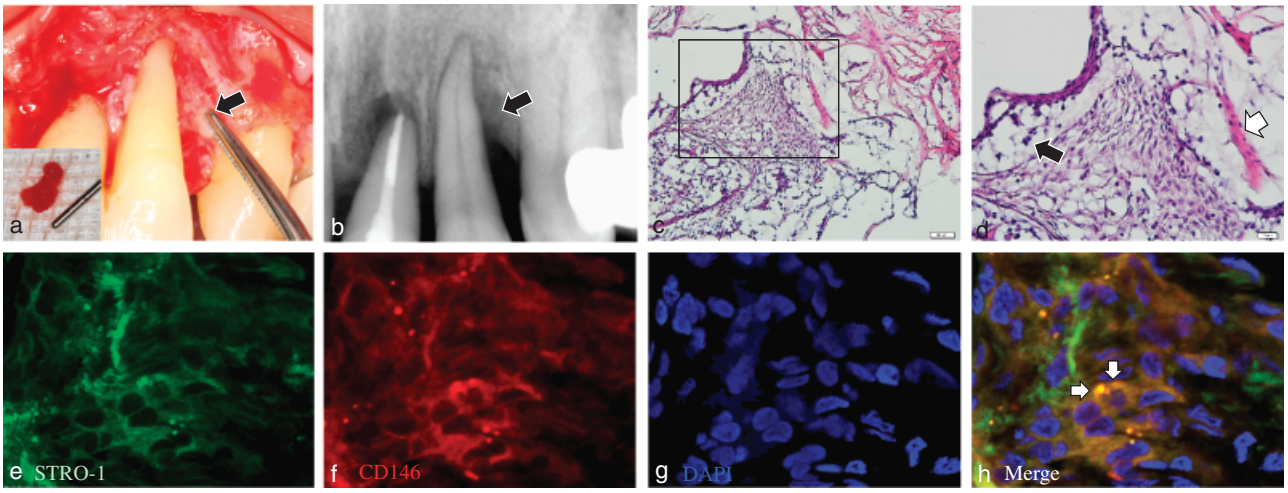

Fig. 1. Acquisition of inflamed human periodontal ligament stem cells and histological/immunohistochemical observation. (a) Clinical photographs showing the acquisition of inflamed tissue from an intra-bony defect. The arrow shows the granulation tissue obtained from periodontal intra-bony defect. After careful debridement, inflamed periodontal tissue (3 × 3 mm) was obtained from the bottom of intra-bony defect (inset). (b) Radigraphic photograph showing the corresponding intra-bony defect. (c) Histological staining (haematoxylin and eosin) reveals infiltration of inflammatory cells and relatively healthy fibrous tissues mixed together. Box represents the area of higher magnification. Original magnifications: × 100. (d) Higher magnification clearly distinguishes infiltration of inflammatory leucocytes (arrow) from relatively healthy fibroblasts in fibrous tissues (open arrow). Original magnifications: × 400. (e–h) Immunohistochemical analysis using double staining under confocal light scanning microscopy showed that surface antigen STRO-1- and CD146-positive cells are clearly observed throughout the obtained inflamed tissue. Surface antigens were stained with fluorescein isothiocyanate (STRO-1, green) and Texas red (CD146, red), and the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, blue). Arrows indicate the positively stained cell surface markers in merged image.

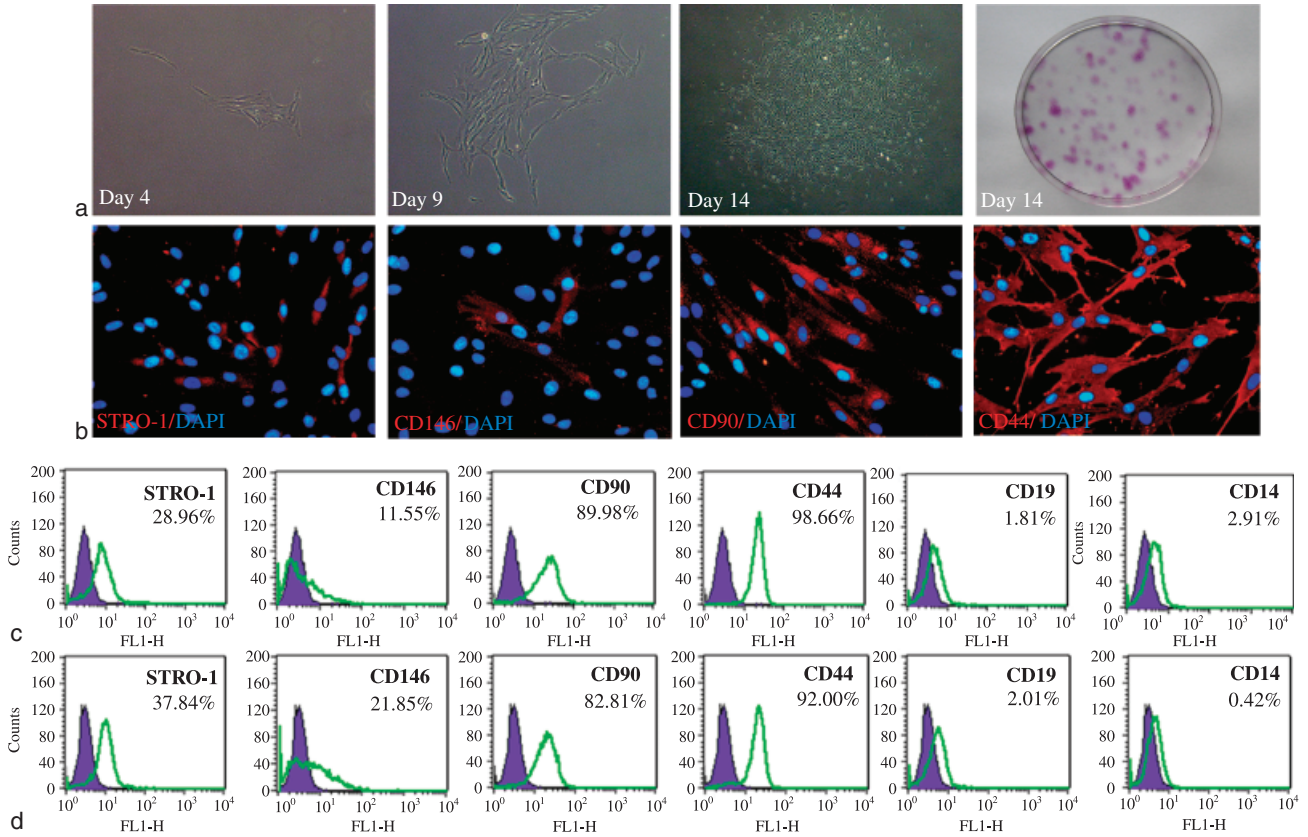

Fig. 2. Characterization of inflamed human periodontal ligament (PDL) stem cells (ihPDLSCs) and comparison with hPDLSCs. (a) Cell proliferation of ihPDLSCs at 4, 9, and 14 days after seeding. Spindle-like cells were observed, and colonies were confirmed after 14 days (stained by crystal violet). (b) Immunocytochemical staining showing that the cultured ihPDLSCs expressed STRO-1, CD146, CD90, and CD44. Surface antigens were stained with Cy3 (red) and the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, blue). Immunophenotype analysis of ihPDLSCs (c) and hPDLSCs (d) by flow cytometry. Cultured ihPDLSCs and hPDLSCs were incubated with specific monoclonal antibodies against the cell-surface marker antigens STRO-1, CD146, CD90, CD44, CD19, and CD14, followed by florescence conjugated secondary antibodies. Both ihPDLSCs and hPDLSCs showed similar expression of surface antigens.

ing that the isolated ihPDLSCs did indeed originate from the PDL tissue (Fig. 3g).

Proliferative and migratory activities of ihPDLSCs

After demonstrating the basic characteristics of MSCs, whether the presence of inflammation had affected the proliferative and migratory potentials of ihPDLSCs was investigated using various methods to compare those potentials. First, the proliferative potential of ihPDLSCs was assessed relative to that of hPDLSCs using the MTT assay, which is a basic method used to test cell viability and proliferation. The findings revealed a generally similar proliferation profile during the observation period (Fig. 4a). There were significant difference between ihPDLSCs and hPDLSCs on days 7 and 9, but they became similar again with no significant difference after day 12. Second, the BrdU labelling efficacy of ihPDLSCs was analysed by BrdU incorporation, which takes place during the S phase of the cell cycle, indicating that the cells are actively replicating their DNA and proliferating. It was found that the degree of BrdU uptake did not differ significantly between ihPDLSCs and hPDLSCs, as illustrated in Fig. 4b. Third, cell-cycle analysis by DNA staining to further compare ihPDLSCs and hPDLSCs was performed in duplicate. The percentages of ihPDLSCs and hPDLSCs did not differ significantly in the G1 ($66.4 \pm 2.2\%$ *versus* $65.3 \pm 1.2\%$, respectively), S ($28.4 \pm 1.5\%$ *versus* $30.2 \pm 1.7\%$, respectively), and G2/M ($6.4 \pm 1.7\%$ *versus* $5.2 \pm 3.7\%$, respectively) phases. Based on these data, we concluded that the proliferative potential of ihPDLSCs was similar to that of hPDLSCs.

Whether inflammatory changes affect the migratory potential of PDL stem cells was investigated by seeding and culturing hPDLSCs and ihPDLSCs separately onto dishes, marking their initial position with a scratch (Fig. 4d) and then measuring the distance the cells migrated every 24 h using an image-analysis programme. By 72 h after scratching, ihPDLSCs (1.36 ± 0.09 mm) had migrated significantly further (by approximately 1.4 times) than the hPDLSCs (0.99 ± 0.17 mm; $p < 0.05$). Cell-migration assays revealed that the migratory activity of ihPDLSCs was thus significantly enhanced compared with that of hPDLSCs.

Cementum and PDL regeneration by transplanted ihPDLSCs

Because ihPDLSCs might represent a potential source of cells for tissue engineering, the regenerative potential of ihPDLSCs for cementum and related PDL tissue was evaluated in an in vivo ectopic transplantation model. Clinical healing was generally uneventful, and

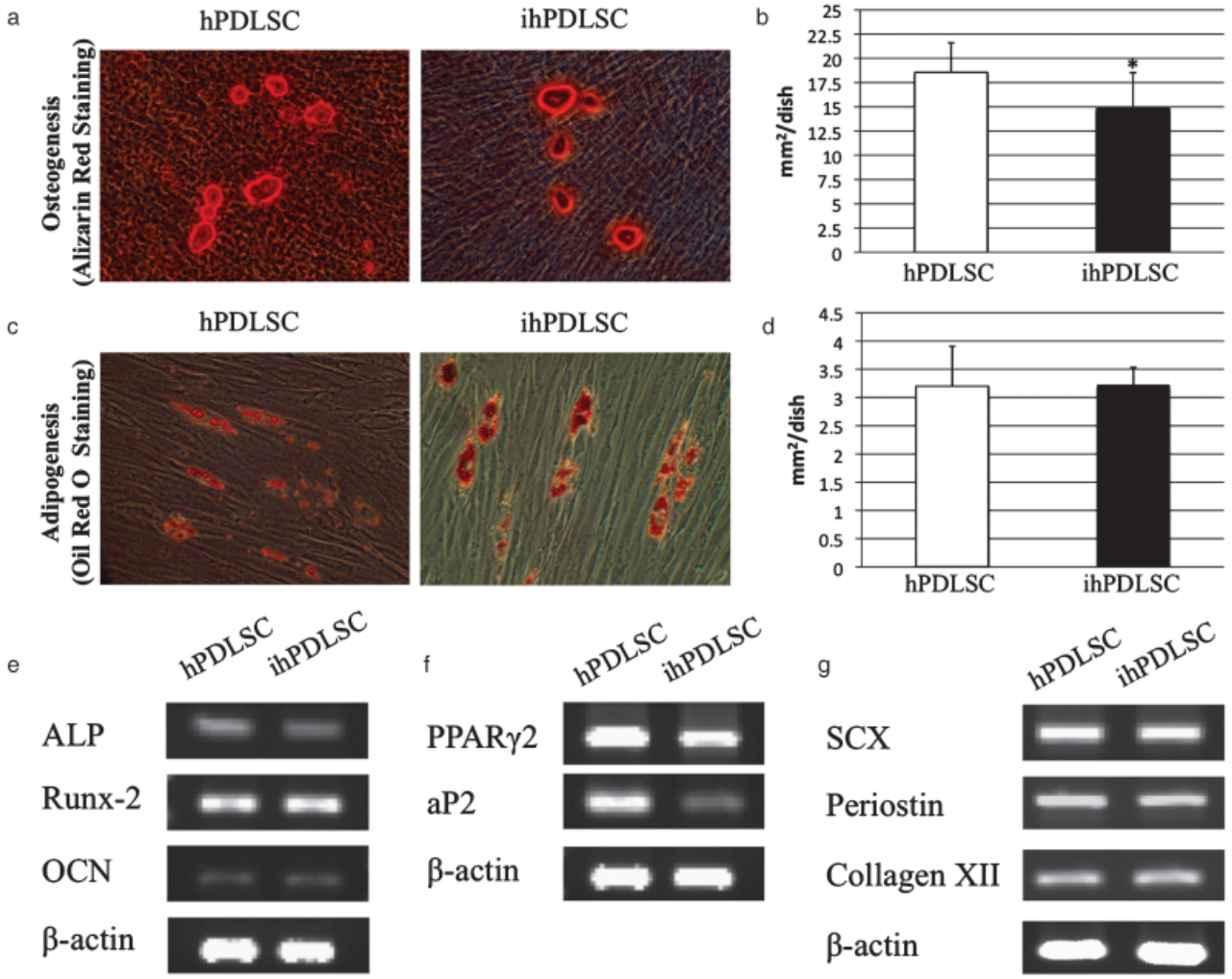

Fig. 3. Osteogenic and adipogenic differentiations of inflamed human periodontal ligament (PDL) stem cells (ihPDLSCs) and hPDLSCs. (a) Mineralized nodules formed after 4 weeks of osteogenic induction (stained with alizarin red). (b) The total area of mineralized nodules were compared and there was statistically significant decrease in total area of mineralized nodules in ihPDLSCs ($^{*}p < 0.05$). (c) The results of adipogenic induction of ihPDLSCs after 2 weeks (stained with oil red O) are shown. (d) The lipid vacuole formations were compared between ihPDLSCs and hPDLSCs, and the total area was similar without statistical significance. (e, f) Osteogenic and adipogenic differentiation-related mRNA expression. Both osteogenic and adipogenic mRNAs of ihPDLSCs and hPDLSCs were highly expressed. (g) PDL-related mRNA including scleraxis, periostin, and collagen XII were highly expressed in ihPDLSCs and hPDLSCs demonstrating that the obtained MSCs were originated from PDL tissues. β-actin is shown as a control for RNA sample quality. ALP, alkaline phosphatase; OCN, osteocalcin; aP2, adipocyte protein 2; PPARγ2, peroxisome proliferator-activated receptorγ2, SCX, scleraxis.

material exposure or complications at the surgical site were not observed. The newly formed cementum-like tissue comprised mineralized tissue that was in direct contact with the carrier. New cementum was formed along the surface of the carrier, and there was evidence of cementogenic activity in the form of a cementoblast-like cell lining. Overall, the newly formed cementum appeared to be cellular cementum, inside which some cementocytes were entrapped, and the newly formed cementum exhibited the characteristics of highly mineralized tissue when viewed with the aid of polarized light microscopy. Interestingly, the total area of newly formed cementum was significantly smaller in the ihPDLSC group compared with the hPDLSC group (Fig. 5b), and this was consistent with in vitro results of osteogenic differentiation (Fig. 3b). ihPDLSCs were able to form a dense PDL tissue around the newly formed cementum. Dense collagen fibres that were organized in parallel and with a high degree of cellular invasion were also associated with newly formed cementum (as evidenced by Picrosirius staining). More importantly, polarized microscopy revealed that Sharpey's fibres running inside the cementum tissue with an apparent fibre insertion pattern. Immunohistochemical analysis revealed the presence of hMito^{+} cells along the surface of the newly formed cementum and in the PDL tissue, confirming that the transplanted ihPDLSCs had provoked the regenerative potential of the transplanted human cells. The histological results demonstrate that the ihPDLSCs had successfully regenerated new cementum-like tissue and PDL fibres, showing that ihPDLSCs isolated from the granulation tissue of periodontal defects retain the

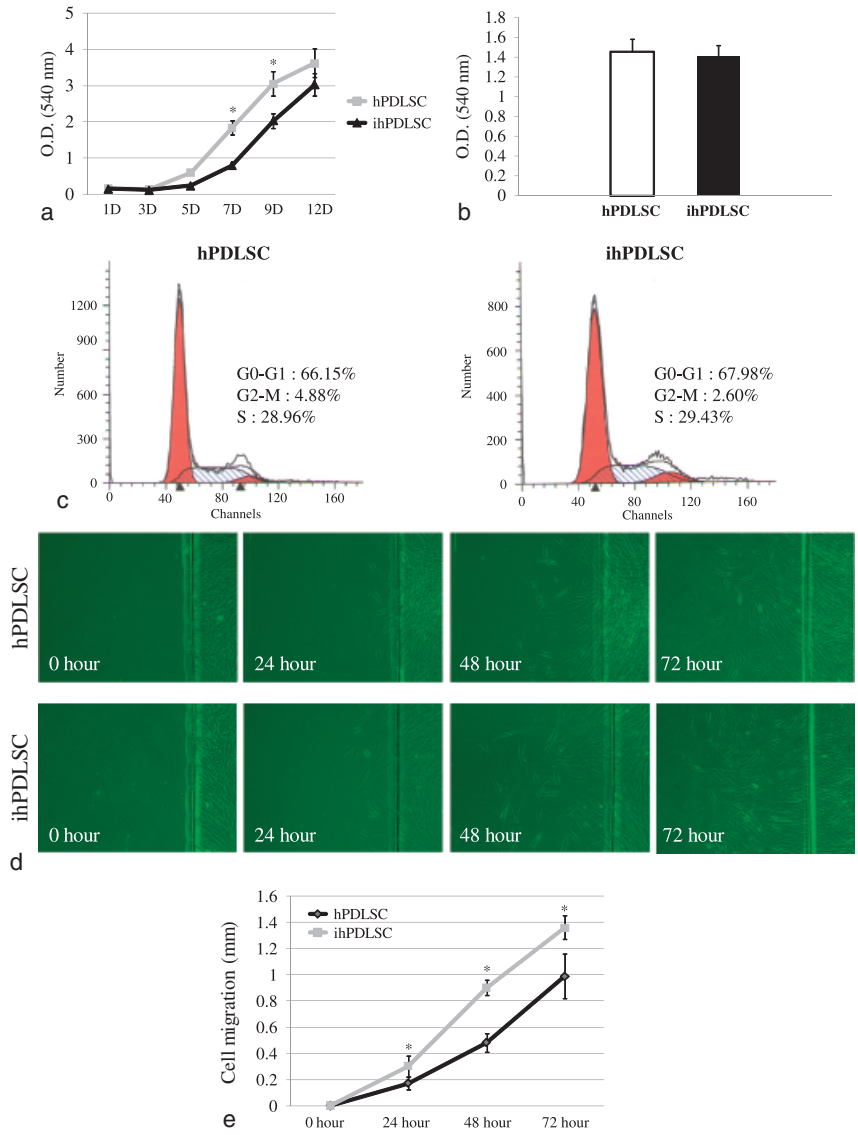

Fig. 4. Cell proliferation and migration, as assessed by the MTT assay (a), bromodeoxyuridine (BrdU) uptake (b), cell-cycle analysis (c), and cell scratch assay (d, e). (a) MTT assays were performed over 12 days. Human periodontal ligament (PDL) stem cells (hPDLSCs) and inflamed human PDL stem cells (ihPDLSCs) showed similar patterns of increases in absorbance at 540 nm; there was no significant difference between the two groups except on days 7 and 9. (b) BrdU uptake results. The total amount of BrdU uptake was similar in hPDLSCs and ihPDLSCs (no statistically significant difference). (c) Representative data of cell-cycle analysis showing similar patterns of cell cycling for hPDLSCs and ihPDLSCs. (d) Representative figures showing cell migration of hPDLSCs and ihPDLSCs at 0, 24, 48, and 72 h after scratching. Note that the cell migration of ihPDLSCs was outside the field of magnification at 72 h. (e) The distance achieved by cell migration was measured using an image-analysis program. A statistically significant difference was observed between the two groups after 24–72 h of migration. (*$p < 0.05$). OD, optical density.

regenerative potential for cementum-like tissue and PDL fibres.

Discussion

For the present study we hypothesized that MSCs reside in periodontitis-affected PDLs, and demonstrated that these MSCs can be isolated and expanded ex vivo. We confirmed that these cells possess the basic characteristics of MSCs and also compared the proliferative and migratory potentials of hPDLSCs and ihPDLSCs. More importantly, we were able to demonstrate that theses ihPDLSCs retain the capacity to produce cementum-like tissues and PDL fibres when transplanted ex vivo.

There are conflicting results on the effects of aging on hPDLSCs, but it was reported recently that age influences the proliferation and differentiation potential of hPDLSCs (Zheng et al. 2009). Ideally, the characteristics of ihPDLSC and hPDLSCs should be compared between age-matched groups, but there are practical difficulties to compare these groups because chronic periodontitis is dominantly observed in elders while orthodontic treatment and extraction of wisdom tooth are common in the young. The present study was mainly focused on the evaluation of the alternate source for MSCs in regard to conventional source, and the age of donors were not matched. It should be further elucidated in the future study whether ihPDLSCs exhibit different features when isolated from young and aged donors, and it would be important to compare these features with the commercially available MSCs of age- and sex-matched groups for positive control (Gay et al. 2007, Iwata et al. 2010).

There are currently various known sources for MSCs of dental origin, including the wisdom teeth, or healthy teeth that have been extracted for orthodontic purposes. However, tooth extraction can sometimes be technically difficult and can cause severe discomfort to patients. Furthermore, the extraction of wisdom teeth and healthy teeth for orthodontic purposes is not a common treatment option compared with periodontal treatment. The prevalence of periodontal disease around the world reportedly lies in the range 5–20% (Albandar & Rams 2002), and periodontal involvement is frequently observed in everyday clinical situations. Therefore, there are more chances to access inflamed PDL tissue than PDL tissue from extracted healthy teeth. To date, it has been considered a prerequisite to remove the granulation tissue from periodontal defects resulting from periodontitis. The rationale behind this clinical procedure is that the inflammatory characteristics of the granulation tissue may be an obstacle for cells in the adjacent undamaged PDL spaces with regenerative potential, preventing them from repopulating previously infected defects. Granulation tissue is thus believed to deter any healing potential of periodontal tissue (Lindhe &

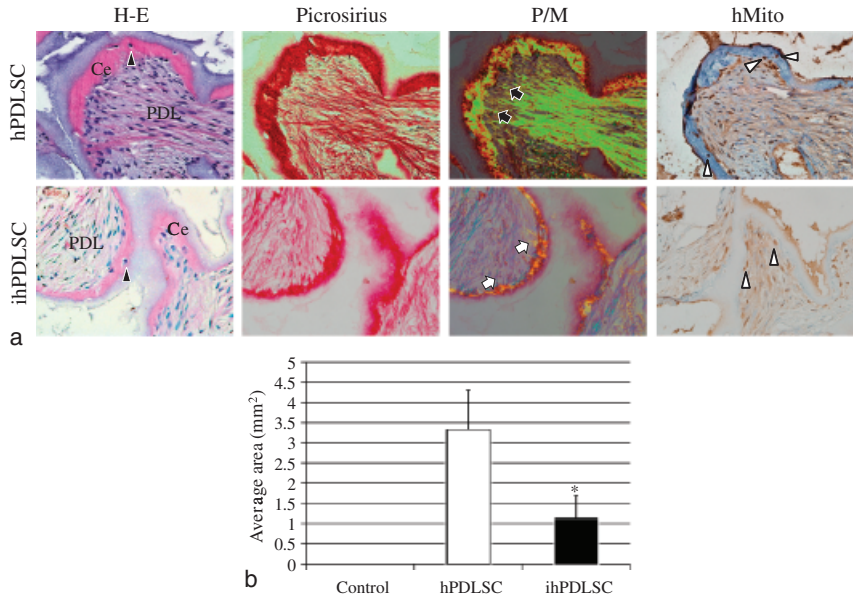

Fig. 5. Histological and immunohistochemical analysis of ex vivo ectopic transplantation of human periodontal ligament (PDL) stem cells (hPDLSCs) and inflamed human PDL stem cells (ihPDLSCs). (a) Cementum-like structures formed (Ce) with cementoblast-like cells incorporated inside the newly formed mineralized tissue (arrows in left column) after 8 weeks of transplantation (HE, haematoxylin and eosin). Picrosirius staining and polarized light microscopy (P/M) reveal collagen fibres running in parallel, with fibre insertion into cementum-like tissue like Sharpey's fibres (open arrows, arrows in right central column). Cementoblast-like cells entrapped within in cementum-like tissue were positive for anti-human-specific mitochondria antibody staining (hMito; arrow heads in right column). (b) Measurement of the area of newly formed cementum. The total area of newly formed cementum was significantly increased for ihPDLSCs than for hPDLSCs ($^{*}p < 0.05$). No mineralized tissue was observed in the cell-free control group.

Nyman 1985). However, our findings show that the granulation tissue of periodontal defects contains a significant population of multipotent MSCs that can be isolated and expanded under particular experimental conditions. These results are in line with the previous report in which inflamed periapical granulation tissue contains the potential to differentiate into osteogenic cells that are responsible for periapical tissue regeneration (Maeda et al. 2004). Given these data, the diseased tissue that is discarded during routine periodontal flap surgery might provide an alternative source of cells for tissue engineering, and may represent an easily accessible source of MSCs, with increased acceptance from patients and fewer ethical complications.

The isolation and characterization of PDLSCs from inflamed periodontal sites have been reported previously (Chen et al. 2006, Widera et al. 2007), but whether the inflammatory stimuli cause changes in the proliferation or migration of PDLSCs was not established. We therefore evaluated and compared the proliferative potential of ihPDLSCs and hPDLSCs using MTT and BrdU-uptake assays, and cell-cycle analysis using DNA staining. Our results demonstrate that the proliferative potentials of healthy and inflamed PDL stem cells are almost equivalent in vitro. It was demonstrated previously that the treatment of well-characterized proinflammatory cytokines (tumour necrosis factor-α, interleukin-1β) resulted in an up-regulation of cytokine secretion from MSCs (Zhukareva et al. 2010). However, little if any information is available on the proliferative changes in MSCs under inflammatory conditions. Based on the current results, we can assume that the proliferative potential of PDL stem cells remains unchanged under inflammatory conditions. Further studies are required to fully evaluate the differences in proliferative potential between hPDLSCs and ihPDLSCs.

On the basis of the finding that MSCs have the potential for migration and tissue-specific homing in response to several injury-related and pathologic conditions (Azizi et al. 1998, Sasaki et al. 2008), we postulated that ihPDLSCs isolated from granulation tissue have an altered migratory potential, because the abundance of inflammatory mediators inside granulation tissue might have mobilized or attracted hPDLSCs from healthy PDL tissue (Spaeth et al. 2008, Fu et al. 2009). Tondreau et al. (2009) recently, investigated the role of cytokines on the migration of bone-marrow-derived MSCs and demonstrated that the migration of these cells was enhanced in response to interleukin-6 and platelet-derived growth factor subunit bb. Other than that, limited information is available regarding the effects of inflammatory stimuli on the migration of ihPDLSCs. However, an increase in the stem-cell population was identified in the extravascular area as well as near the cementum of the PDL of periodontitis-affected teeth, whereas small clusters of putative stem cells were limited in the paravascular region in the PDL of healthy teeth (Chen et al. 2006), suggesting that paravascular cells are recruited for periodontal repair, as observed in the wounded mouse PDL (Gould et al. 1980, McCulloch 1985) and inflamed monkey gingiva (Nemeth et al. 1993). Given these data, it can be suggested that inflammatory changes in the periodontium influence the migratory activity and distribution of ihPDLSCs in some way. The present study found that the migratory activity of ihPDLSCs was significantly greater than that of hPDLSCs. However, it is not clear, at this point, whether the migratory activity of ihPDLSCs had changed genetically by inflammatory stimulations in comparison with hPDLSCs. Future studies should include analysis of the precise mechanism underlying the regulation of the migratory activity of ihPDLSCs by the microenvironment, and their possible genetic change.

In the present study, a typical cementum-like/PDL structure was regenerated in the ectopic transplantation model of both hPDLSCs and ihPDLSCs. The mineralized tissue appeared to be distinctively different from the typical bone and bone marrow tissue shown in previous reports (Seo et al. 2004), and apparent running of Sharpey's fibres with insertion into cementum tissue was observed. However, the degree of ihPDLSC-induced cementum regeneration was

significantly lower than that induced by hPDLSCs, which was consistent with the results of in vitro mineralized nodule formation. The presence of inflammation inhibited the osteoblastic differentiation of human MSCs (Gilbert et al. 2000, Li & Makarov 2006), and DPSCs from inflamed dental pulp also exhibited decreased mineralization potential (Alongi et al. 2010). The current understanding regarding this mechanism is that MSCs isolated from inflamed tissues are arrested at the early stage of differentiation unless specific stimulation for differentiation is not provided (Maeda et al. 2004); our results are in line with these findings. However, the exact molecular mechanism for this phenomena including genetic changes should be addressed in future studies. Collectively, our results demonstrate that isolated ihPDLSCs retain the potential to regenerate cementum-like tissues and PDL fibres, but with decreased cementogenic potential, and the molecular mechanism underlying this process remains to be elucidated.

Conclusion

The present study has demonstrated that a population of hPDLSCs that express the characteristics of MSCs resides within the granulation tissue of periodontitis-affected intra-bony defects. These cells have the potential to regenerate new cementum and related PDL tissue under ex vivo conditions. The findings of the present study suggest that hPDLSCs obtained from inflamed tissues within periodontal defects during periodontal flap surgery are an alternative and easily accessible source of multipotent adult stem cells for use in clinical and research use without the need to extract third molars or to access the dental pulp. This newly described ihPDLSC population within periodontal defects might thus represent a promising source of cells for therapeutic use.

References

Albandar, J. M. & Rams, T. E. (2002) Global epidemiology of periodontal diseases: an overview. *Periodontology 2000* **29**, 7–10.

Alongi, D. J., Yamaza, T., Song, Y., Fouad, A. F., Romberg, E. E., Shi, S., Tuan, R. S. & Huang, G. T. (2010) Stem/progenitor cells from inflamed human dental pulp retain tissue regeneration potential. *Regenerative medicine* **5**, 617–631.

Azizi, S. A., Stokes, D., Augelli, B. J., DiGirolamo, C. & Prockop, D. J. (1998) Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats – similarities to astrocyte grafts. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 3908–3913.

Chen, S. C., Marino, V., Gronthos, S. & Bartold, P. M. (2006) Location of putative stem cells in human periodontal ligament. *Journal of Periodontal Research* **41**, 547–553.

Cortellini, P., Prato, G. P. & Tonetti, M. S. (1999) The simplified papilla preservation flap. A novel surgical approach for the management of soft tissues in regenerative procedures. *International Journal of Periodontics and Restorative Dentistry* **19**, 589–599.

Fortier, L. A. (2005) Stem cells: classifications, controversies, and clinical applications. *Veterinary Surgery* **34**, 415–423.

Fu, X., Han, B., Cai, S., Lei, Y., Sun, T. & Sheng, Z. (2009) Migration of bone marrow-derived mesenchymal stem cells induced by tumor necrosis factor-alpha and its possible role in wound healing. *Wound Repair and Regeneration* **17**, 185–191.

Fujii, S., Maeda, H., Wada, N., Tomokiyo, A., Saito, M. & Akamine, A. (2008) Investigating a clonal human periodontal ligament progenitor/stem cell line in vitro and in vivo. *Journal of Cellular Physiology* **215**, 743–749.

Gay, I. C., Chen, S. & MacDougall, M. (2007) Isolation and characterization of multipotent human periodontal ligament stem cells. *Orthodontics and Craniofacial Research* **10**, 149–160.

Gilbert, L., He, X., Farmer, P., Boden, S., Kozlowski, M., Rubin, J. & Nanes, M. S. (2000) Inhibition of osteoblast differentiation by tumor necrosis factor-alpha. *Endocrinology* **141**, 3956–3964.

Gould, T. R., Melcher, A. H. & Brunette, D. M. (1980) Migration and division of progenitor cell populations in periodontal ligament after wounding. *Journal of Periodontal Research* **15**, 20–42.

Gronthos, S., Brahim, J., Li, W., Fisher, L. W., Cherman, N., Boyde, A., DenBesten, P., Robey, P. G. & Shi, S. (2002) Stem cell properties of human dental pulp stem cells. *Journal of Dental Research* **81**, 531–535.

Horiuchi, K., Amizuka, N., Takeshita, S., Takamatsu, H., Katsuura, M., Ozawa, H., Toyama, Y., Bonewald, L. F. & Kudo, A. (1999) Identification and characterization of a novel protein, periostin, with restricted expression to periosteum and periodontal ligament and increased expression by transforming growth factor beta. *Journal of Bone and Mineral Research* **14**, 1239–1249.

Iwata, T., Yamato, M., Zhang, Z., Mukobata, S., Washio, K., Ando, T., Feijen, J., Okano, T. & Ishikawa, I. (2010) Validation of human periodontal ligament-derived cells as a reliable source for cytotherapeutic use. *Journal of Clinical Periodontology* **37**, 1088–1099.

Karimbux, N. Y., Rosenblum, N. D. & Nishimura, I. (1992) Site-specific expression of collagen I and XII mRNAs in the rat periodontal ligament at two developmental stages. *Journal of Dental Research* **71**, 1355–1362.

Kerkis, I., Kerkis, A., Dozortsev, D., Stukart-Parsons, G. C., Gomes Massironi, S. M., Pereira, L. V., Caplan, A. I. & Cerruti, H. F. (2006) Isolation and characterization of a population of immature dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers. *Cells Tissues Organs* **184**, 105–116.

Li, X. & Makarov, S. S. (2006) An essential role of NF-kappaB in the ''tumor-like'' phenotype of arthritic synoviocytes. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 17432–17437.

Lindhe, J. & Nyman, S. (1985) Scaling and granulation tissue removal in periodontal therapy. *Journal of Clinical Periodontology* **12**, 374–388.

Maeda, H., Wada, N., Nakamuta, H. & Akamine, A. (2004) Human periapical granulation tissue contains osteogenic cells. *Cell and Tissue Research* **315**, 203–208.

McCulloch, C. A. (1985) Progenitor cell populations in the periodontal ligament of mice. *Anatomical Record* **211**, 258–262.

Melcher, A. H. (1976) On the repair potential of periodontal tissues. *Journal of Periodontology* **47**, 256–260.

Miura, M., Gronthos, S., Zhao, M., Lu, B., Fisher, L. W., Robey, P. G. & Shi, S. (2003) SHED: stem cells from human exfoliated deciduous teeth. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 5807–5812.

Nemeth, E., Kulkarni, G. W. & McCulloch, C. A. (1993) Disturbances of gingival fibroblast population homeostasis due to experimentally induced inflammation in the cynomolgus monkey (*Macaca fascicularis*): potential mechanism of disease progression. *Journal of Periodontal Research* **28**, 180–190.

Roberts, W. E. & Jee, W. S. (1974) Cell kinetics of orthodontically-stimulated and non-stimulated periodontal ligament in the rat. *Archives of Oral Biology* **19**, 17–21.

Sasaki, M., Abe, R., Fujita, Y., Ando, S., Inokuma, D. & Shimizu, H. (2008) Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type. *Journal of Immunology* **180**, 2581–2587.

Seo, B. M., Miura, M., Gronthos, S., Bartold, P. M., Batouli, S., Brahim, J., Young, M., Robey, P. G., Wang, C. Y. & Shi, S. (2004) Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* **364**, 149–155.

Shi, S. & Gronthos, S. (2003) Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *Journal of Bone and Mineral Research* **18**, 696–704.

Sonoyama, W., Liu, Y., Fang, D., Yamaza, T., Seo, B. M., Zhang, C., Liu, H., Gronthos, S., Wang, C. Y., Wang, S. & Shi, S. (2006) Mesenchymal stem cell-mediated functional tooth regeneration in swine. *PLoS One* **1**, e79.

Sonoyama, W., Liu, Y., Yamaza, T., Tuan, R. S., Wang, S., Shi, S. & Huang, G. T. (2008) Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. *Journal of Endodontics* **34**, 166–171.

Spaeth, E., Klopp, A., Dembinski, J., Andreeff, M. & Marini, F. (2008) Inflammation and tumor microenvironments: defining the migratory itinerary of mesenchymal stem cells. *Gene Therapy* **15**, 730–738.

Tondreau, T., Meuleman, N., Stamatopoulos, B., De Bruyn, C., Delforge, A., Dejeneffe, M., Martiat, P., Bron, D. & Lagneaux, L. (2009) In vitro study of matrix metalloproteinase/tissue inhibitor of metalloproteinase production by mesenchymal stromal cells in response to inflammatory cytokines: the role of their migration in injured tissues. *Cytotherapy* **11**, 559–569.

Widera, D., Grimm, W. D., Moebius, J. M., Mikenberg, I., Piechaczek, C., Gassmann, G., Wolff, N. A., Thevenod, F., Kaltschmidt, C. & Kaltschmidt, B. (2007) Highly efficient neural differentiation of human somatic stem cells, isolated by minimally invasive periodontal surgery. *Stem Cells and Development* **16**, 447–460.

Zheng, W., Wang, S., Ma, D., Tang, L., Duan, Y. & Jin, Y. (2009) Loss of proliferation and differentia-

tion capacity of aged human periodontal ligament stem cells and rejuvenation by exposure to the young extrinsic environment. *Tissue Engineering. Part A* **15**, 2363–2371.

Zhukareva, V., Obrocka, M., Houle, J. D., Fischer, I. & Neuhuber, B. (2010) Secretion profile of human bone marrow stromal cells: donor variability and response to inflammatory stimuli. *Cytokine* **50**, 317–321.

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Clinical Relevance

Scientific rationale for the study: The presence of putative stem cells within the PDL tissue was demonstrated. However, there is little report on the isolation of stem cells from inflamed PDL tissue. In this study, we isolated and characterized human PDL stem cells from inflamed PDL tissue, and evaluated their regenerative potential.

Principal findings: Human PDL stem cells were successfully isolated from inflamed periodontal tissue, and retained characteristics of mesenchymal stem cells and regenerative potential for cementum and related PDL tissue.

Practical implications: Inflamed human PDL tissue can potentially provide an alternate source of mesenchymal stem cells for regenerative treatment.