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Putative stem cells in regenerating human periodontium

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Background and Objective: Human postnatal stem cells have been identified in periodontal ligament, with the potential to regenerate the periodontium *in vivo*. However, it is unclear if periodontal ligament stem cells are present in regenerating periodontal tissues. The aim of this study was to identify and localize putative stem cells in block biopsies and explant cultures of regenerating human periodontal tissues.

Material and Methods: Guided tissue regeneration was carried out on the molars of three human volunteers. After 6 wk, the teeth with the surrounding regenerating tissues and bone were surgically removed and processed for immunohistochemistry. The mesenchymal stem cell-associated markers STRO-1, CD146 and CD44 were used to identify putative stem cells. Cell cultures established from regenerating tissue explants were analysed by flow cytometry to assess the expression of these markers. Mineralization, calcium concentration and adipogenic potential of regenerating tissue cells were assessed and compared with periodontal ligament stem cells, bone marrow stromal stem cells and gingival fibroblasts.

Results: STRO-1$^+$, CD44$^+$ and CD146$^+$ cells were identified in the regenerating tissues. They were found mainly in the paravascular and extravascular regions. Flow cytometry revealed that cultured regenerating tissue cells expressed all three mesenchymal stem cell associated markers. The regenerating tissue cells were able to form mineral deposits and lipid-containing adipocytes. However, the level of mineralization in these cells was lower than that of periodontal ligament stem cells and bone marrow stromal stem cells.

Conclusion: Cells with characteristics of putative mesenchymal stem cells were found in regenerating periodontal tissues, implying their involvement in periodontal regeneration.

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Periodontitis is a disease of the periodontium that is characterized by the irreversible loss of connective tissue attachment and supporting alveolar bone (1). For many decades, periodontists have been interested in regenerating the tissues destroyed by periodontitis. Successful regeneration requires the coordination of many events at both cellular and molecular levels (2–4). Only periodontal ligament cells appear to possess the ability to re-establish lost connective tissue attachment (5,6). The periodontal ligament cells are a heterogeneous population (7–9) and may include progenitor or stem cells that facilitate the restoration of lost periodontal tissues.

Stem cells are defined as cells that have the capacity to self-renew and give rise to differentiated progeny (10). These cells can be broadly divided into two categories: immortalized pluripotent embryonic stem cells that can form all tissue types; and adult somatic stem cells, such as bone marrow haematopoietic stem cells and mesenchymal stem cells, which have more restricted differentiation and proliferation potentials.

Identification of mesenchymal stem cells in human tissues has relied on the expression of various cell-surface markers based on immunohistochemical staining and flow cytometric analysis (11). In particular, human mesenchymal stem cells have been identified using STRO-1 antibody and antibodies against cell adhesion molecule CD146 and vascular cell adhesion molecule-1 (CD106) (12–15). In addition, these cells also express CD44 antigen, a marker found on many mesenchymal cell types (14,15). Individually, these markers may be insufficient to identify progenitor cells because of potential cross-reactivity with other cell types. However, the use of various combinations of these (comprising at least three out of the four stated markers) can provide a more effective means to identify putative mesenchymal stem cells in human tissues (16).

Mesenchymal stem cells isolated from human periodontal ligament have been termed periodontal ligament stem cells (14,17). Periodontal ligament stem cells appear to be the precursors of synthetic cells (e.g. fibroblasts, osteoblasts and cementoblasts) with the multipotent capacity to generate adipocytes, osteoblast-like and cementoblast-like cells *in vitro*, as well as cementum-like and periodontal ligament-like tissues when transplanted into immunocompromised mice (14,17,18). In addition to their developmental potential, there is evidence that periodontal ligament stem cells may participate in the healing of periodontal defects in animals (14,19,20). However, to date there is no evidence demonstrating the existence of periodontal ligament stem cells in regenerating periodontal defects in humans, or the involvement of these cells in human periodontal wound healing. In light of this, the aim of our study was to investigate whether periodontal ligament stem cells participate in the regeneration of human periodontal defects. Block biopsies of teeth and the surrounding tissues derived from the regenerating human periodontal human defect were obtained as previously described (21,22) and periodontal ligament stem cells were identified *in situ* by immunohistochemical staining with STRO-1, anti-CD146 and anti-CD44 antibodies. In addition, we compared the differentiation potential of cells derived from biopsies of regenerating tissue with that of periodontal ligament stem cells, bone marrow stromal stem cells and gingival fibroblasts derived from normal and non-related donors. We hypothesized that periodontal ligament stem cells are present in regenerating periodontal tissues and demonstrate a differentiation potential classically associated with mesenchymal stem cells.

Material and methods

Sampling and surgical procedure

This study comprised three systemically healthy patients (two men, aged 43 and 82 years, and one woman, aged 24 years), attending the Oral and Maxillofacial Surgery Unit of the Adelaide Dental Hospital for extractions of class II furcation-involved molar teeth as part of their overall dental treatment plan. Informed consent to participate in this study was obtained from all three individuals in accordance with the research protocol approved by the University of Adelaide Human Research Ethics Committee (H-125-2005). After local anaesthesia was administered, sulcular incisions were made and full-thickness mucoperiosteal flaps were raised adjacent to the buccal surface of the molar tooth. The granulation tissue was removed from the furcation defect and the tooth surface was thoroughly debrided before placing an expanded polytetrafluoroethylene Gore-Tex® membrane (W. L. Gore and Associates, Flagstaff, AZ, USA). Wound closure was achieved by flap repositioning coronal to the cemento–enamel junction. Postoperatively, the patients were reviewed at 1 and 3 wk for suture removal and oral hygiene maintenance. After a 6-wk healing period, a block biopsy containing the molar tooth and surrounding tissues (regenerating tissue and alveolar bone) was sampled in two patients for immunohistochemical staining. In the third patient, the molar tooth was extracted and the associated regenerating tissue was excised for cell culture.

Tissue preparation

Immediately after the biopsy was taken, the blocks of fresh teeth, regenerating tissues and surrounding bone were fixed in 10% neutral-buffered formalin for 48 h and then decalcified in 5% formic acid for 6 to 8 wk. Complete decalcification of tooth and bone was verified by using standard radiographic examination. The decalcified samples were then dehydrated in ascending ethanol solutions, cleared with histolene and embedded in paraffin. Serial sections of 5 μm were cut, mounted on charged SuperFrost® Plus slides (Menzel GmbH and Corporation, Braunschweig, Germany) and dried in an oven at 60°C for 20 min.

Primary antibodies

The antibodies used for immunostaining and flow cytometry were mouse monoclonal immunoglobulin STRO-1 (IgM) and immunoglobulins reactive to human CD146 (clone CC9, IgG2a), CD44 (clone H9H11, IgG1) and the pericyte marker, 3G5 antigen (IgM). Corresponding isotype-matched immunoglobulins of irrelevant specificity, 1A6.12 (IgM), 1D4.5 (IgG2a) and 1B5 (IgG1) were used as negative controls. The STRO-1, anti-CD44 and anti-CD146 antibodies were available in house. The 3G5 antibody was obtained from the American Tissue Culture Collection (Manassas, VA, USA). All isotype-matched controls were generously provided by Prof. L. K. Ashman, Medical Science Building, University of Newcastle (NSW, Australia).

Immunohistochemistry

The immunostaining process was performed at room temperature (approx. 23°C) unless otherwise stated. The sections were deparaffinized in histolene, rehydrated in descending ethanol solutions and then rinsed with distilled water. Antigen retrieval was performed on sections prior to STRO-1 or CD146 staining by pretreating with sodium citrate buffer (10 mM, pH 6) at 80°C for 30 min. Sections were then cooled

for 30 min and washed in phosphate-buffered saline (pH 7.4). No antigen retrieval was required for CD44 staining. Endogenous peroxidase activity was quenched in all sections by the addition of 0.5% hydrogen peroxide in methanol (BDH Chemicals, Poole, UK) for 30 min. After rinsing in phosphate-buffered saline, sections were incubated in 3% normal horse serum for a further 30 min to block nonspecific binding. The primary antibodies were diluted to give optimal staining (STRO-1, 1:4; anti-CD146, 1:4000; and anti-CD44, 1:4) and the sections were incubated overnight in a humidity chamber. Control sections were incubated with isotype-matched immunoglobulin at the following dilutions: 1A6.12 (1:4), 1D4.5 (1:4000) and 1B5 (1:4).

After incubation, the sections were rinsed with phosphate-buffered saline, and a secondary biotinylated horse-anti-mouse IgG or IgM (Vector Laboratories, Burlingame, CA, USA) was applied for 30 min. Sections were then rinsed with phosphate-buffered saline and incubated with streptavidin peroxidase conjugate (Pierce Biotechnology, Rockford, IL, USA) for 1 h. Colour development was visible 7 min after the addition of buffered diaminobenzidine substrate (Pierce Biotechnology). The sections were rinsed with phosphate-buffered saline, counterstained with Mayer's haematoxylin, dehydrated in ascending alcohol solutions, cleared with histolene and mounted with cover slips using Aquamount PIX mounting medium (BDH Chemicals). The presence and localization of STRO-1^+, $CD146^+$ and $CD44^+$ cells in the regenerating tissues were determined using a Nikon Microphot-FXA light microscope (Nikon Corporation, Tokyo, Japan), and photomicrographs were taken with a digital camera (Nikon D1; Nikon Corporation).

Cell isolation and culture

From one of the biopsies (taken from a 24-year-old woman) a primary cell culture was established from an explant of the regenerating soft connective tissue. The regenerating tissue was digested in collagenase I (3 mg/mL; Worthington Biochemical, Lakewood, NJ, USA) and dispase II (4 mg/mL; Roche Diagnostic/Boehringer Mannheim, Indianapolis, IN, USA) at 37°C for 2 h to obtain a single-cell suspension, as previously described (23). The regenerating tissue cells were then seeded into six-well plates (2.2×10^5 cells/well; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and cultured in α-modified minimal essential medium (SAFC Biosciences, Lenexa, KS, USA), supplemented with 10% foetal bovine serum (JRH Biosciences, Lenexa, KS, USA), 2 mM L-glutamine (SAFC Biosciences), 100 μM L-ascorbate-2-phosphate (WAKO Industries, Tokyo, Japan), 1 mM sodium pyruvate (SAFC Biosciences), 50 U/mL of penicillin and 50 μg/mL of streptomycin (Sigma-Aldrich, St Louis, MO, USA) in a humidified atmosphere (37°C, 5% CO_2). Periodontal ligament stem cells, bone marrow stromal stem cells and human gingival fibroblasts derived from normal non-related donors were isolated and cultured as previously described (12,14,24). Culture media was replenished twice weekly. Upon reaching confluence, the cells were passaged by trypsinization (0.05% trypsin/ethylenediaminetetra-acetic acid solution; Gibco/Invitrogen Corporation, Grand Island, NY, USA) and transferred to tissue culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) for further expansion. Confluent monolayers of regenerating tissue cells, periodontal ligament stem cells, bone marrow stromal stem cells and human gingival fibroblasts were viewed under phase-contrast microscopy (Olympus CKX41; Olympus Corporation, Tokyo, Japan).

Flow cytometric analysis

Flow cytometric analysis was performed as previously reported (13). Briefly, regenerating tissue cells were trypsinized and resuspended in blocking buffer containing Hank's balanced salt solution (JRH Biosciences) supplemented with 5% foetal bovine serum (JRH Biosciences), 1% bovine serum albumin (ICN Biomedicals, Aurora, OH, USA) and 5% normal human serum (Australian Red Cross, Adelaide, SA, Australia) for 1 h on ice to block nonspecific binding. A total of 2×10^5 cells were incubated with STRO-1, CD146, CD44 or 3G5 antibody (20 μg/mL), or with nonimmune isotype-matched mouse antibody controls, for 1 h on ice. After washing twice with Hank's balanced salt solution (JRH Biosciences) containing 5% foetal bovine serum (JRH Biosciences) for 2 min, the cells were incubated with secondary fluorescein isothiocyanate-conjugated goat anti-mouse IgM or IgG (1:50 dilution; Southern Biotechnology Associates, Birmingham, AL, USA), for an additional 45 min on ice. The cells were then fixed in 1% formalin, 2% D-glucose and 0.01%

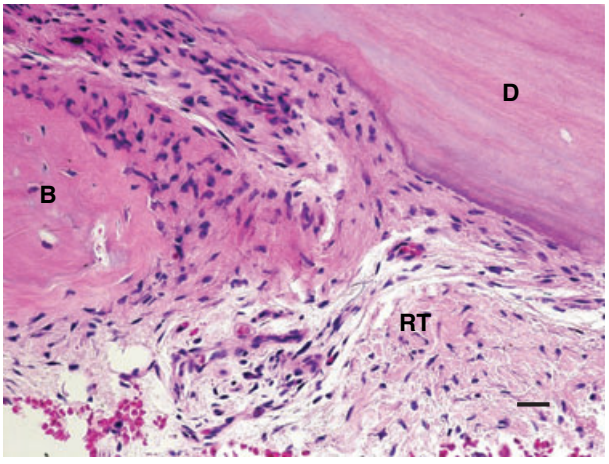

Fig. 1. Haematoxylin and eosin staining of regenerating periodontal tissues (RT), periodontal ligament, dentine (D), cementum, and alveolar bone (B). Scale bar, 40 μm.

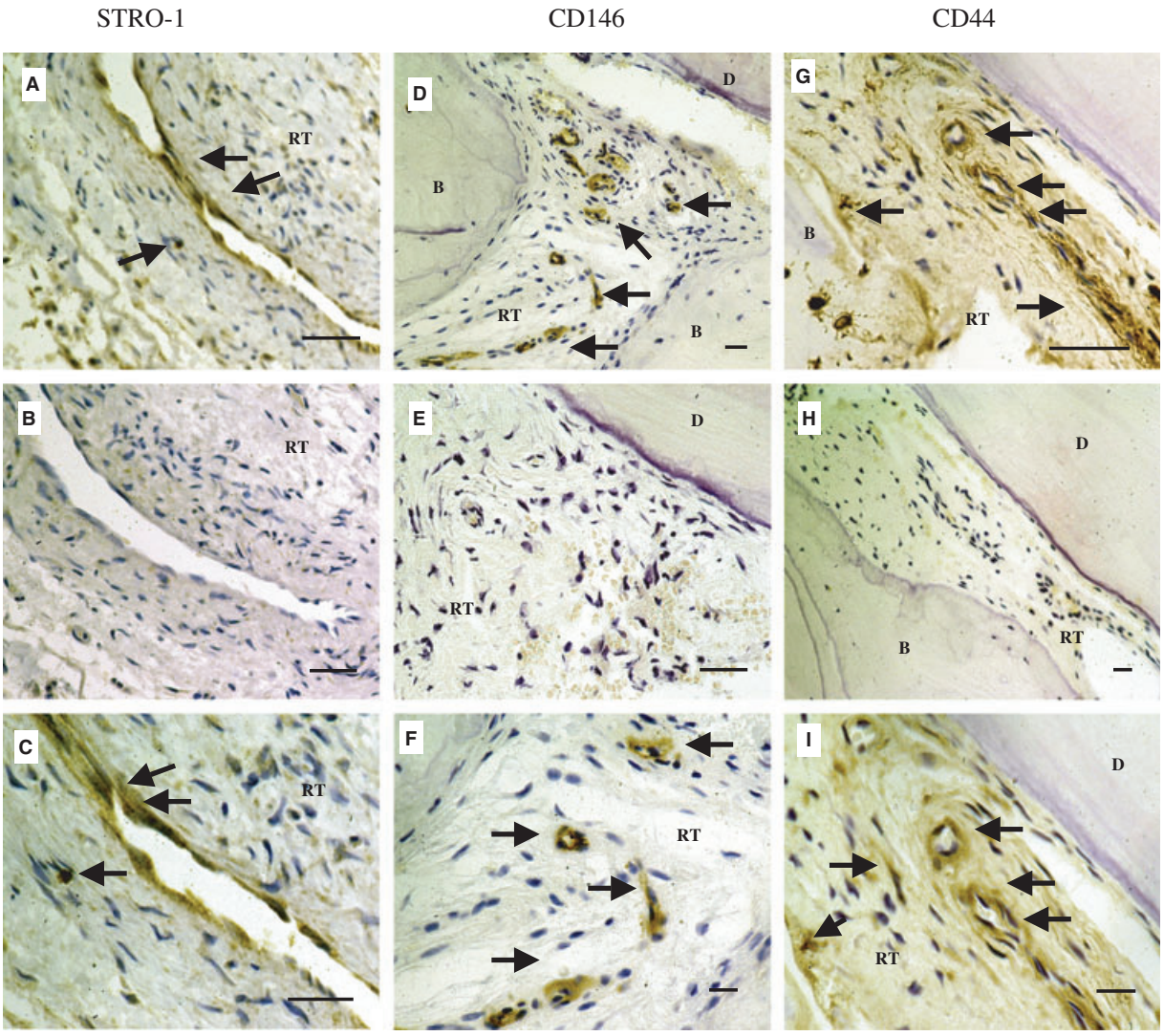

Fig. 2. (A) STRO-1 staining (arrow) of regenerating periodontal tissue and (B) its isotype-matched control (×20 magnification). Scale bar, 40 μm. (C) STRO-1 staining of fresh regenerating periodontal tissues at higher magnification (×40). Scale bar, 10 μm. (D) CD146 staining (arrow) of regenerating periodontal tissue and (E) its isotype-matched control (×20 magnification). Scale bar, 40 μm. (F) CD146 staining of fresh regenerating periodontal tissue at higher magnification (×40). Scale bar, 10 μm. (G) CD44 staining (arrow) of regenerating periodontal tissue (×40 magnification) and (H) its isotype-matched control (×20 magnification). Scale bar, 40 μm. (I) CD44 staining of fresh regenerating periodontal tissue at higher magnification (×40). Scale bar, 10 μm. B, alveolar bone; D, dentine; PDL, periodontal ligament; RT, regenerating periodontal tissues.

sodium azide in phosphate-buffered saline. The fluorescence intensity of cells was analysed using a Cytomics FC-500 flow cytometer (Beckman Coulter, Miami, FL, USA) and Cytomics CXP analysis version 2.0 software (Beckman Coulter).

In vitro differentiation

The potential of regenerating tissue cells to differentiate into fibroblasts, osteoblasts, as well as adipocytes, was investigated to verify that these cells had a differentiation capacity similar to that previously reported for periodontal ligament stem cells (14). Cells derived from the regenerating tissues in their fifth passage in culture were used for the differentiation assays. For comparative purposes, cultures of periodontal ligament stem cells, bone marrow stromal stem cells and human gingival fibroblasts derived from normal non-related donors were used between the fifth and seventh passage as positive and negative controls. It has previously been demonstrated that both periodontal ligament stem cells and bone marrow stromal stem cells have the ability to form mineral or fat deposits under specific *in vitro* conditions (12,14). In contrast, human gingival fibroblasts do not possess this differentiation potential (24). Cells were initially cultured in normal growth media (as previously described) for 24 h to enable cell attachment and subsequently induced in mineralization

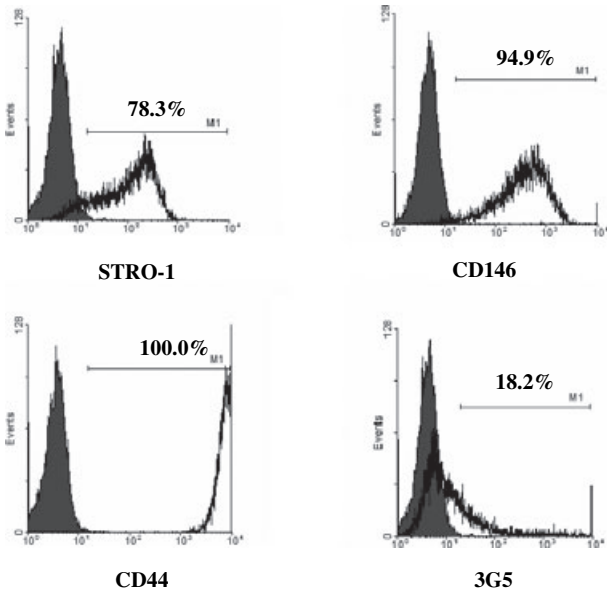

Fig. 3. Analysis of single-cell suspensions of regenerating tissues for STRO-1, CD146, CD44 and 3G5 expressions by single-colour flow cytometry. The (black) plots represent the proportion of cells with STRO-1, CD146, CD44 and 3G5 antibody binding relative to that of (red) the isotype-matched negative-control antibodies, 1A6.12, 1D4.5 and 1B5.

media containing α-minimal essential medium modification supplemented with 5% fetal bovine serum (JRH Biosciences), 10^{-5} M dexamethasone (David Bull Laboratory, Sydney, NSW, Australia), 10 mM Hepes (JRH Biosciences), 1.8 mM KH_2PO_4 (BDH Chemicals), 2 mM L-glutamine (SAFC Biosciences), 100 μM L-ascorbate-2-phosphate (WAKO Industries), 50 U/mL of penicillin and 50 μg/mL of streptomycin (Sigma-Aldrich) (25). Adipogenesis was induced in α-minimal essential medium modification supplemented with 5% fetal bovine serum, 0.5 nM isobutylmethylxanthine (Sigma-Aldrich), 0.5 μM hydrocortisone (Royal Adelaide Hospital Pharmacy, Adelaide, South Australia), 60 μM indomethacin (Sigma-Aldrich), 2 mM L-glutamine (SAFC Biosciences), 1 mM sodium pyruvate (SAFC Biosciences), 100 μM L-ascorbate-2-phosphate (WAKO Industries), 50 U/mL of penicillin and 50 μg/mL of streptomycin (Sigma-Aldrich), as described previously (26). All cells were incubated at 37°C in 5% CO_2 and fed twice weekly. After 4 wk, mineral accumulation was detected by staining with 2% Alizarin Red S (pH 4.2) and lipid deposits were identified by staining with Oil Red O. Mineral deposition or lipid formation was identified as distinct red staining under phase-contrast microscopy (Olympus Corporation). For quantitative assessment of mineralization, the extracellular calcium concentration for each cell type (regenerating tissue cells, periodontal ligament stem cells, bone marrow stromal stem cells and human gingival fibroblasts) was assessed in quadruplicate in a 48-well plate. The calcium concentration was determined using a calcium assay kit (Sigma-Aldrich) and the average calcium concentration was calculated in mmol/L per 10^5 cells. Because this study was intended to be descriptive in nature, statistical analysis of differences in calcium concentration between different cell lines was not conducted.

Results

Haematoxylin and eosin staining

Standard haematoxylin and eosin staining of cross-sections of the regenerating periodontal tissues demonstrated the general architecture of the tissues recovered from underneath the guided tissue regeneration (GTR) membrane. This had the general appearance of moderately cellular fibrous connective tissue that was closely apposed to both the alveolar bone and cementum surfaces (Fig. 1).

Immunohistochemistry: STRO-1

Staining of regenerating periodontal tissues with the STRO-1 antibody (Figure 2A) showed that positively stained cells were sparsely distributed around the paravascular and extravascular areas of the periodontal ligament, in contrast to the isotype-matched control (Fig. 2B). STRO-1^+ cells morphologically appeared as either elongated cells with oval-shaped nuclei and extended cytoplasm, or as round cells with intensely stained nuclei and a small cytoplasm (Fig. 2C).

Immunohistochemistry: CD146

Staining of regenerating periodontal tissues with CD146 antibody (Fig. 2D) showed that positively stained cells were present around the paravascular and extravascular areas of the periodontal ligament, in contrast to the isotype-matched control (Fig. 2E). Interestingly, the pattern of staining appeared to mimic cell migration through the tissue. Similar to STRO-1^+ cells, $CD146^+$ cells were either elongated or round in morphology (Fig. 2F). The elongated cells had oval-shaped nuclei and a large cytoplasm, whereas the round cells had intensely stained nuclei and a small cytoplasm.

Immunohistochemistry: CD44

Sections stained for CD44 displayed a greater degree of staining compared with STRO-1- and CD146-stained sections as a result of differences in the relative specificity of each marker for progenitor cells. Whilst CD44 expression has previously been associated with mesenchymal stem cells, it should be noted that CD44 has also been reported to be expressed by a wide range of other cell types. Staining of regenerating periodontal tissues with CD44 antibody (Fig. 2G) showed that positively stained cells were distributed around the paravascular region of the periodontal ligament, the extravascular spaces and the

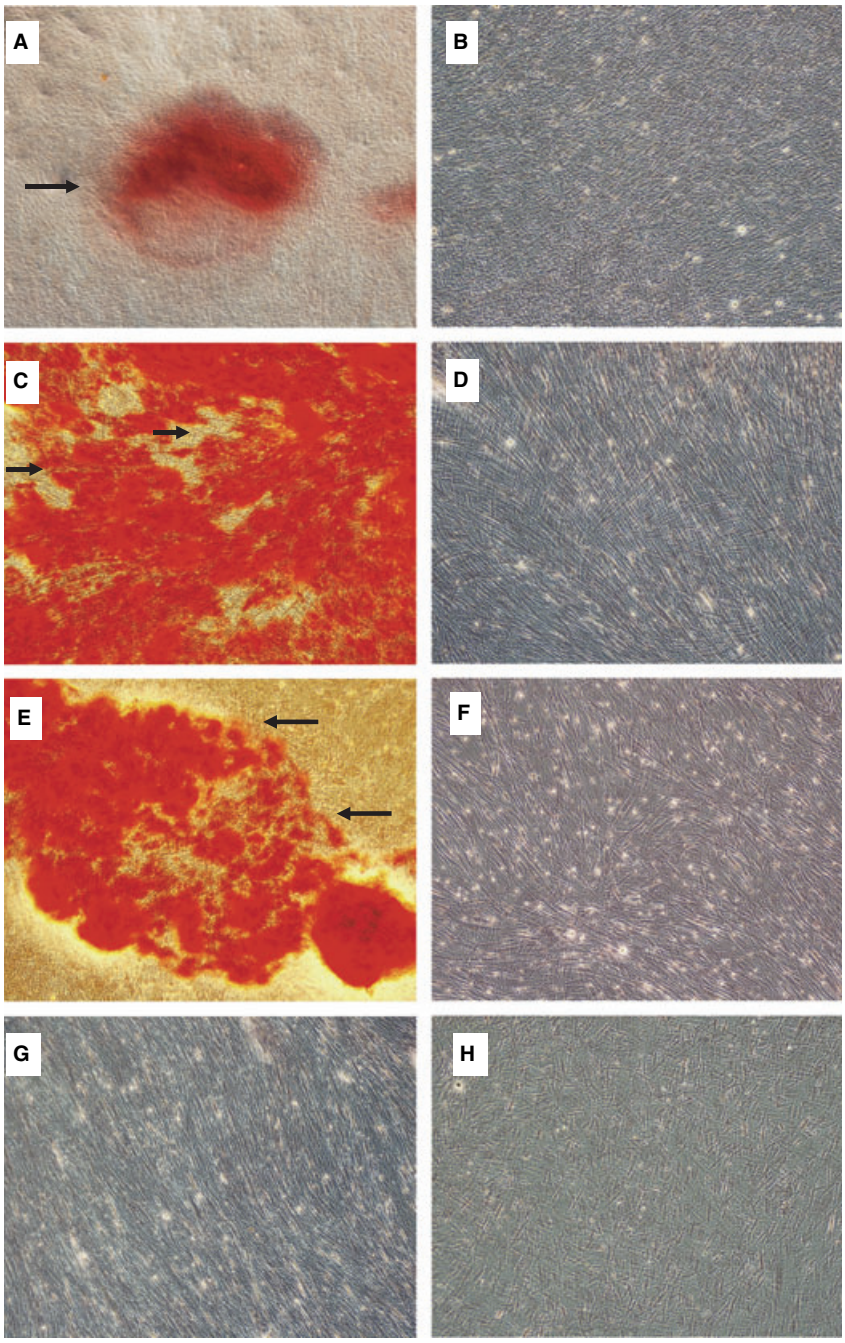

Fig. 4. Mineralized deposits stained with Alizarin Red solution (arrows) in cultures of (A) regenerating tissue cells in induction media and (B) in noninduction media; (C) periodontal ligament stem cells in induction media and (D) in noninduction media; (E) bone marrow stromal stem cells in induction media and (F) in noninduction media; and (G) human gingival fibroblasts in induction media and (H) in noninduction media after 4 wk of induction *in vitro* (×10 magnification).

periphery of the periodontal ligament near the alveolar bone, in contrast to the isotype-matched control (Fig. 2H). $CD44^+$ cells had an elongated morphology (Fig. 2I) with oval-shaped nuclei and an extended cytoplasm.

Flow cytometry

Characterization of the immunophenotype of cultured regenerating tissue cells by flow cytometry revealed binding of STRO-1, anti-CD146 and anti-CD44 antibodies (Fig. 3) in the majority of cells relative to the isotype-matched control antibodies 1A6.12, 1D4.5 and 1B5, respectively. This observation indicated that the antigen reactive to STRO-1 and CD146 was expressed by regenerating tissue cells. However, expression of the pericyte-associated marker, 3G5, only occurred in a small percentage of regenerating tissue cells.

Differentiation assays

Mineral deposition: Alizarin red staining — In normal culture media, regenerating tissue cells, periodontal ligament stem cells, bone marrow stromal stem cells and human gingival fibroblasts rapidly attached to plastic surfaces and assumed a stellate, fibroblast-like appearance (data not shown). After induction in mineralization medium for 4 wk, Alizarin red mineral deposits were detected in regenerating tissue cell cultures (Fig. 4A), periodontal ligament stem cell cultures (Fig. 4C) and bone marrow stromal stem cell cultures (Fig. 4E), but not in cultures with noninduction media (Fig. 4B,D,F). No positive Alizarin red staining was detected in the gingival fibroblasts cultured in inductive (Fig. 4G) or noninductive (Fig. 4H) media.

Calcium concentration — After 4 wk of induction in mineralization medium, quantitative analysis of extracellular calcium indicated that the average calcium concentration was higher in periodontal ligament stem cell and bone marrow stromal stem cell cultures than in regenerating tissue cultures. No calcium was detected in human gingival fibroblast cultures (Fig. 5).

Adipogenic differentiation — Oil Red O Staining — After 4 wk of induction in adipogenic media, clusters of lipid-containing fat cells were detected in cultures derived from the regenerating tissue (Fig. 6A), periodontal ligament stem cell cultures (Fig. 6C) and bone marrow stromal stem cell cultures (Fig. 6E), but not in cultures with noninduction media (Fig. 6B,D,F). No positive Oil Red O staining was

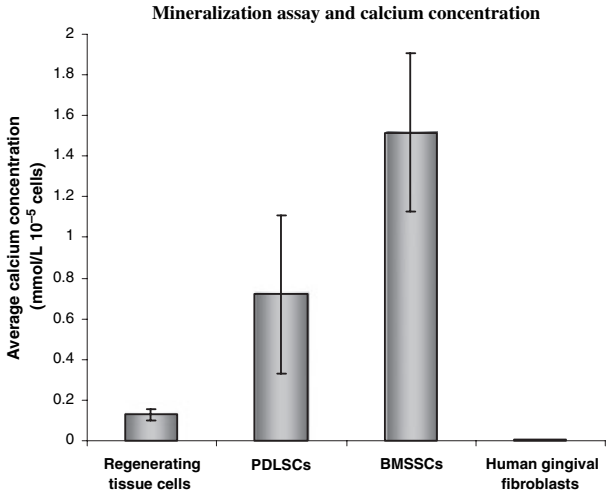

Fig. 5. Graph showing the calcium concentration in regenerating tissue cells, periodontal ligament stem cells, bone marrow stromal stem cells and human gingival fibroblasts after 4 wk of osteo-inductive culture *in vitro*. Histograms and error bars indicate medians and ranges respectively. BMSSCs, bone marrow stromal stem cells; PDLSCs, periodontal ligament stem cells.

detected in the human gingival fibroblasts cultured in inductive (Fig. 6G) or non-inductive (Fig. 6H) media.

Discussion

In this study, cells positively stained with STRO-1, anti-CD146 and anti-CD44 antibodies in the regenerating periodontal tissues demonstrated two distinct morphologies: elongated cells with oval-shaped nuclei and an extended cytoplasm; and round cells with intensely stained nuclei and a small cytoplasm. The appearance of the latter is similar to that of undifferentiated progenitor cells identified from murine (27), ovine (17) and human (14) periodontal ligament and putative mesenchymal stem cells (STRO-1^{BRIGHT}/VCAM-1^+ cells) isolated from human bone marrow (12). The sparse distribution of positively stained cells within the regenerating periodontal tissues in our study is also in concordance with the expected paucity of mesenchymal stem cells in postnatal tissues (28). The location of positively stained cells in our regenerating tissue around the paravascular region or the extravascular region was similar to that reported for periodontal ligament stem cells, bone marrow stromal stem cells and dental pulp stem cells. Furthermore, this observation supports previous studies suggesting a potential vascular origin of mesenchymal stem cells (13,14,16,17,23).

Our findings of both STRO-1^+ and $CD146^+$ cells in regenerating tissues – two markers constitutively, but not exclusively, expressed by perivascular cells and undifferentiated stromal cells (13) – support the notion that some positively stained cells are putative mesenchymal stem cells. A notable feature of the $CD44^+$ staining pattern in regenerating tissues is its wider reactivity compared with STRO-1^+ or $CD146^+$ staining. It is likely that co-expression of the CD44 cell-surface antigen by extracellular components (e.g. collagen, fibronectin, laminin) and other cells in periodontal ligament (e.g. periodontal ligament fibroblasts) (29,30), along with that of putative mesenchymal stem cells, may account for this observation. Therefore, the specificity of CD44 as a marker of mesenchymal stem cells should be questioned because it has been reported to be expressed by many different cell types (31).

Flow cytometric analysis revealed that about 80% or more of regenerating tissue cells in the gated sample were positive for at least one of the STRO-1, CD146 or CD44 markers. A relatively higher expression of CD44 compared with STRO-1 and CD146 can be attributed to the expression of CD44 on both undifferentiated and differentiated cells (30). Nonetheless, the expression of STRO-1 and CD146 markers by the majority of regenerating tissue cells in our study suggests that immature progenitors are also present. Indeed, both the STRO-1 and CD146 markers are expressed by periodontal ligament stem cells (14,15). Furthermore, small proportions of STRO-1^+ and $CD146^+$ cells have been noted to co-express the pericyte-associated antigen, 3G5, where all markers are closely associated with perivascular tissues and, in particular, pericytes (13,15). Our finding that a small proportion of regenerating tissue cells was positive for the 3G5 marker supports previous findings describing 3G5 expression by periodontal ligament stem cells (15) and correlates with the immunohistochemical paravascular localization of STRO-1^+ and $CD146^+$ cells *in situ*. As a result of the potential cross-reactivity of STRO-1 and anti-CD146 antibodies with blood vessel walls (13,29,30), it is also likely that some of the paravascular cells (particularly elongated cells) may represent endothelial cells or smooth muscle cells rather than mesenchymal stem cells. To overcome this problem, we performed a functional assessment of regenerating tissue cells by investigating their differentiation potential *in vitro*.

The differentiation of regenerating tissue cells into osteoblasts and adipocytes was investigated because periodontal ligament stem cells have been shown previously to differentiate into these lineages *in vitro* (i.e. form fat and periodontal ligament-like tissue) (14,17). After 4 wk of mineral and adipogenic induction, we observed that cultured regenerating tissue cells had formed Alizarin Red-positive mineral nodules and Oil Red O-positive lipid-containing adipocytes. The pattern of mineralization and adipogenesis in these cell cultures was similar to that of cultured periodontal ligament stem cells and bone marrow stromal stem cells, implying that progenitor cells or mesenchymal stem cells are likely to be present in regenerating tissues. Interestingly, regenerating tissue cells had a

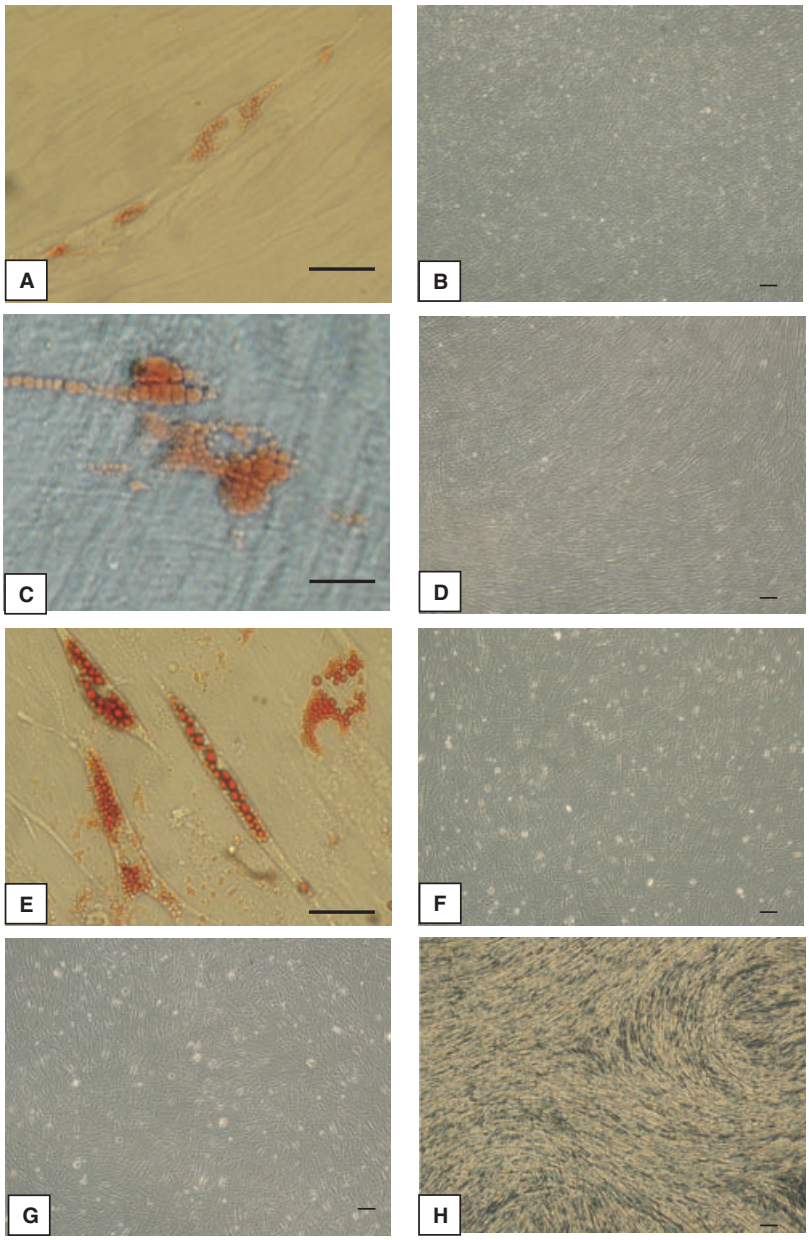

Fig. 6. Adipogenic deposits stained with Oil Red O solution (arrow) in cultures of (A) regenerating tissue cells in induction media and (B) in noninduction media; (C) periodontal ligament stem cells in induction media and (D) in noninduction media; (E) bone marrow stromal stem cells in induction media and (F) in noninduction media; and (G) human gingival fibroblasts in induction media and (H) in noninduction media, after 4 wk of induction *in vitro*. Scale bar, 50 μm.

relatively lower calcium concentration than periodontal ligament stem cells and bone marrow stromal stem cells, and this difference may be explained by the heterogeneous nature of cell populations isolated using the plastic adherence technique (32). This technique has been known to isolate both clonogenic progenitors and committed progeny (33,34). It is likely that the regenerating tissue cells in our study represented a sub-population of cells containing very few progenitor cells, including mesenchymal stem cells, which can account for the less extensive mineralization and the lower calcium concentration in these cells compared with periodontal ligament stem cells and bone marrow stromal stem cells.

The overall findings from immunohistochemical staining, flow cytometric analysis and differentiation assays indicate that some cells derived from human GTR-treated periodontal defects have the immunophenotypical and functional characteristics of periodontal ligament stem cell-like cells. While it is likely that some of these cells are putative stem cells, others may represent partially differentiated cells committed to osteoblastic, fibroblastic or adipocytic lineages, with less growth potential than stem cells. This finding of the presence of putative stem cells in regenerating periodontal tissues in humans is consistent with the reported existence of progenitor cells in the proliferative compartment of wounded periodontal ligament in mice (19,20). As these murine progenitor cells provide a renewable source of cells for periodontal wound healing (35–37), we postulate that periodontal ligament stem cell-like cells in regenerating human periodontal tissues are recruited to the wound site to replenish cells lost in tissue injury and to facilitate periodontal regeneration.

The role of these cells in periodontal wound healing may be comparable with that of dental follicular cells in the developing periodontium (38,39), and their involvement in wound healing provides biological support for the application of periodontal ligament stem cells in regenerative periodontal therapy. Recent studies have demonstrated that a number of cells derived from the dental follicle, periodontal ligament and cementum can all participate in the regeneration of periodontal tissues (40–42). The use of periodontal ligament stem cells with tissue-engineering techniques should constitute a novel strategy for periodontal regeneration (43–46). The potential for further development marks the beginning of a new era in periodontal research and clinical practice.

In conclusion, our findings provide evidence for the presence of putative stem cells in regenerating human periodontal defects. These cells express the three mesenchymal stem cell-associated markers STRO-1, CD146 and

CD44, and have the capacity to form mineral deposits and fat cells *in vitro*. While further work using selected cell populations *in vivo* is required, our findings provide the first evidence that stem cells participate in the healing of regenerating periodontal defects in humans and offer support for the use of stem-cell based tissue engineering in regenerative periodontal therapy.

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