Mesenchymal Stem Cells Acquire Characteristics of Cells in the Periodontal Ligament in vitro

INTRODUCTION

Repair of periodontal ligament appears to involve progenitor cells, present in the periodontal region, capable of forming fibroblasts, osteoblasts, and cementoblasts (Melcher, 1976). These cells appear clustered near blood vessels in the periodontal ligament and have characteristics of early progenitor cells (Gould et al., 1977, 1980; McCulloch, 1985). Moreover, progenitor cells in endosteal spaces potentially migrate from the bone to periodontal ligament to form tissues of the periodontium (McCulloch et al., 1987). Currently, it is unknown if a single progenitor cell type can give rise to all the tissues of the periodontium, but the presence of periodontal progenitor cells is necessary to prevent alveolar bone loss. The repair of periodontal ligament in vitro is a promising approach to investigate the mechanisms of tissue repair and regeneration.

MATERIALS AND METHODS

Mesenchymal stem cells were isolated from bone marrow stroma and cultured in vitro to assess their potential to differentiate into periodontal ligament progenitor cells. The cells were cultured in the presence of osteogenic and periodontal ligament-inducing factors (Braga et al., 1999; Pfeifer et al., 1999). The cells were then subjected to immunohistochemical analysis to determine their expression of osteoblast, osteoclast, and periodontal ligament markers.

RESULTS

Mesenchymal stem cells demonstrated the ability to differentiate into osteoblasts, osteoclasts, and periodontal ligament progenitor cells in vitro. The cells expressed markers specific to each cell type, indicating their potential to contribute to periodontal tissue regeneration.

DISCUSSION

The results suggest that mesenchymal stem cells possess the potential to differentiate into periodontal ligament progenitor cells, providing a new therapeutic approach for periodontal tissue regeneration. Further studies are needed to evaluate the long-term effects of these cells in vivo.

KEY WORDS: mesenchymal stem cell, periodontal ligament, regeneration, repair.

immunosuppressive effects of these cells (Uchida et al. 1998; Bartholomew et al. 2002; Di Nicola et al. 2002). Lack of an immune response suggests a high probability of success for the use of mesenchymal stem cells in clinical protocols. The most recent finding in this area supports and extends this concept by showing that tissue engineering with mesenchymal stem cells has been successful in producing bone (Brow et al. 2002; Partridge et al. 2002).

In our experimental paradigm, mesenchymal stem cells (from male donors) were tagged by fluorescent *in situ* hybridization (FISH) and then stained for a series of proteins that identify cell types present in the periodontium. Changes in mesenchymal stem cell morphology and protein expression were observed in these Y-chromosome-tagged cells (i.e., mesenchymal stem cells) to determine if they differentiated into a specific cell type (e.g., periodontal ligament, from female donors).

**MATERIALS & METHODS**

**Organotypic Tooth Explant Isolation and Culture**

Samples of oral tissue were obtained for clinically necessary extractions with consent of the female human subjects following NIH guidelines and IRB approval. Extracted teeth were rinsed *ex vivo* in phosphate-buffered saline (PBS) containing penicillin and streptomycin and then once in minimal essential medium. The extracted tooth was held in place by a clamp on the crown of the tooth, the apical side facing up. Using a low-speed handpiece and a diamond disk, irrigated with 1X PBS, we removed a section from the extracted tooth at a depth of -0.5 mm (Fig. 1, panels A1 and A2). The section was placed in minimal essential medium supplemented with 10% fetal calf serum and antibiotics and placed in 5% CO₂ atmosphere (Okamoto et al. 1997). Proliferative cells having various morphologies were produced from the section in culture between 7 and 10 days. Cells with periodontal ligament morphology were diluted and cultured to confluence, then plated on glass slides and processed for immunohistochemistry after 24 or more hrs in culture. Human male mesenchymal stem cells were obtained from BioWhittaker Cell Biology Products (Walkersville, MD).

**Figure 1.** Isolation and characterization of periodontal ligament (A1) Cartoon depiction of a parapsial cross-section through the jaw and attached tooth. Tooth slices (rectangle) were made in the apical region of the extracted tooth to avoid any contamination with gingival epithelial cells. Tooth slice, termed organotypic tooth explant (OTE), had dimensions of a half-millimeter depth, a width of 1.4 mm, and a height of 2-5 mm; variation was due to topography of the tooth end differences in circumference. (A2) Paraffin section (12 μm) from an organotypic tooth explant stained with hematoxylin and eosin. Organotypic tooth explants always consisted of cementum (C) and periodontal ligament (PDL) and dentin (D). In approximately 5% of the sections, a layer of alveolar bone (B) was present. (B1) Proliferative periodontal cells were then cultured in vitro from organotypic tooth explants. Greater than 99% of the cells cultured were spindle shaped resembling the morphological properties noted for human periodontal ligament cells (black arrows). (B2) Cultured cells from explants stain positive for collagen III (green fluorescence). Thus, periodontal ligament fibroblasts were isolated from the cellular population based on morphology and high levels of collagen III expression. (C1-G3) Staining of periodontal tissues for collagen III, osteopontin, osteocalcin, BMP-2/4, and bone sialoprotein in vivo was completed on 12-μm paraffin or fresh-frozen sections. Collagen III staining (brown, panel C2), osteopontin staining (brown, panel G2), osteocalcin staining (brown, arrows, panel E2), BMP-2/4 staining (brown, panels F1 and F2), and bone sialoprotein staining (brown, arrows, panels G2 and G3) of osteogenotypic tooth explants. Panels C1, D1, E1, and G1 are controls where non-immune serum was added, instead of primary antibody, during the immunohistochemical procedure. In panels C1-G2, the bone (b), periodontal ligament (pdl), cementum (c), and dentin (d) tissue layers are indicated. Bars = 50 μm (B2, C1, C2, D1, G1, D2), 100 μm (E1, E2, G2), and 200 μm (F1). Panels F2 and G3 are high-magnification images of regions in panels F1 and G2, respectively.
USA) and cultured according to the manufacturer's directions in MSCRM medium provided by the company. Undifferentiated cells are guaranteed between 2 and 4 passages. Criteria for undifferentiated mesenchymal stem cells are that the cells must be negative for blood cell or hematopoietic progenitor cell markers CD11b, CD34, and CD45, plus express markers indicative of mesenchymal stem cells CD105, CD166, CD29, and CD44 (Pietragalla et al., 1999; Le Blanc et al., 2003) and have the ability to differentiate into osteogenic, chondrogenic, and adipogenic lineages by means of specific growth medium (BioWhittaker Cell Biology Products). Mesenchymal stem cells were used in slides or mixed with periodontal cells isolated from the argenetic tooth explants at ratios of 1:1, 2:1, and 10:1. Individual cell types and cocultures were then plated in minimal essential medium supplemented with 10% fetal calf serum and antibiotics. Note: Allowing the ratio of the 2 cell types did not have any observaive effect on the differentiation events. Co-cultures of cells were isolated after 0, 7, 14, and 21 days and processed for immunohistochemistry and in situ hybridization. Experiments were repeated at least three times with at least two different donors with similar results.

**Immunohistochemistry**

Frozen or paraffin sections (30 μm) from tooth explants or cells, cultured on slides, were fixed (4% formaldehyde in 1X PBS) for 30 min. Explants were decalcified in 0.5 M EDTA (pH 6.8) for more than 10 days before being sectioned. The sections were blocked (10% normal goat serum or 3% bovine serum albumin) in 1X PBS and incubated in primary antibody > 12 hrs at 4°C. Primary antibodies used for staining included a goat polyclonal antibody (1:200 dilution) to BMP-2 (R&D Systems, Minneapolis, MN, USA) and rabbit polyclonal antibodies (Pentola Labs, San Carlos, CA, USA) (1:500, monoclonal antibodies specific for collagen III (Chemicon, Temecula, CA, USA) 1:50, osteopontin (MabPep, 10, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) 1:50, tenascin ( clone WW/D1-9C5, Developmental Studies Hybridoma Bank) 1:5. Slides were then incubated with biotinylated goat anti-rabbit or goat anti-mouse (1:500, Vector Labs, Burlington, CA, USA) or rabbit anti-goat horseradish peroxidase (Biomedical, Forest Grove, OR, USA). Biotinylated secondary incubations were followed by streptavidin-horseradish peroxidase (Vectastain ABC kit, Vector Labs). Detection of a signal was completed by use of the DAB or AEC kit following the manufacturer's directions (Vector Labs) and cells were counter-stained with hematoxylin and mounted. Bright-field images were captured with a DC290 digital camera (Easonan Kodak, Rochester, NY, USA).

**Double-immunohistochemistry and in situ Hybridization**

Fixed cells were processed for immunohistochemistry as described above, except that signal detection was completed with use of the USA-kit1 biotinylated antibody signal amplification kit following the manufacturer's directions (Perkin-Elmer Life Sciences Inc., Wallacula, MA, USA). Note: The primary antibody dilutions were increased by a factor of 2 in fluorescent labeling procedures (e.g., 1:5 becomes 1:10). Following the signal detection step, the slides were incubated in proteinase K (20 μg/ml) in PBS and 0.1% SDS at 37°C for 30-60 sec rinsed in 1X PBS, and fixed for 10 min followed by 3 rinses in 1X PBS, 5 min each. A Y-chromosome-specific probe (Manez et al., 2000), random-prime-labeled with digoxigenin-11-dUTP following the manufacturer's directions (Roche Diagnostics, Indianapolis, IN, USA), was mixed with hybridization buffer (50% formamide, 10% dextran sulfate, 0.02 M Tris, pH 7.4, 0.4 M NaCl, 1X Denhardt's solution). The slides were coated, coverslipped, sealed with rubber cement, and heated to 95°C for 5 min (http://intranarul.nlm.nih.gov/lcn/rgg/Protocols/echrom.html). After an overnight incubation at 55°C, the slides were rinsed in 1X PBS, incubated with anti-DIG-POD (Roche Diagnostics), rinsed, and reacted with the SA-10 HRP, then Covase, and 3,3-diaminobenzidine (DAB) to reveal labelling. Controls included cells processed for immunohistochemistry, using 10% normal goat serum in 1X PBS with addition of a primary antibody or without reacting with tyramide or processing for ISH without the addition of a Y chromosome probe. Fluorescent images were captured with a CCD camera model 782-v (Princeton Instruments, Princeton, NJ, USA). Images for staining localized within the cell were captured with an upright Leica TCS-P2 confocal microscope equipped with argon, krypton, and helium neon lasers with excitation wavelengths of 488, 546, and 633 μm, respectively. Leica confocal software was used to compile a series of images made through the interior of the cells, thus providing a cross-sectional image.

**Quantification of Immunostaining**

Fluorescent SA-488 staining of various proteins in the different cultures was captured on the CCD camera and quantitated by means of MetaMorph software (Universal Imaging Corporation, West Chester, PA, USA). For each experiment, all slides were processed simultaneously for a specific antibody, so that homogeneity in the staining procedure would be ensured between samples. After capture of the images at the same magnification, the threshold was set and maintained for each slide in the experiment, and the optical density was calculated by use of the morphometric analysis function within the software package. The optical density values from separate experiments with mesenchymal cells from two different donors and periodontal ligament cells from at least two different donors were combined. Optical density values were given as the mean ± SEM. Statistical significance was determined by one-way ANOVA, and comparison of different cultures was completed by means of the Mann-Whitney test.

**RESULTS**

Identification of Periodontal Ligament

Immunohistochemical staining of sections made from organotypic tooth explants (Fig. 1, A1 and A2) indicated that adult periodontal ligament, cultured from the explants (Fig. 1, B1), expressed collagen III (Fig. 1, B2). Moreover, these cells had a spindle or spindle-like morphology (Fig. 1, B1). Collagen III (Fig. 1, C1, C2), osteopontin (Fig. 1, D2), osteocalcin (Fig. 1, E2), and BMP-24 (Fig. 1, F1 and F2) were also expressed in explants taken acutely, thus representing in vitro expression. Collagen III staining was restricted to the periodontal ligament and was not detected in the cementum and dentin layers (Fig. 1, C2). Osteopontin staining was present in the periodontal ligament, and low-intensity staining was observed in the cementum (Fig. 1, D2). Osteocalcin staining was heterogeneous in the periodontal ligament (arrows), and staining was observed in the bone, dentin, and cementum (Fig. 1, E2). BMP-24 could be detected in the periodontal ligament, cementum, and bone tissue (Fig. 1, F1 and F2).

Bone
sialoprotein was present in the cementum, dentin, and alveolar bone (Fig. 1, G2 and G3). In contrast, periodontal ligament did not stain for bone sialoprotein (Fig. 1, G2 and G3). Non-immune serum dilutions showed no staining (Fig. 1, C1, D1, E1, and G1). Thus, cellular morphology—plus a pattern of high collagen III expression, the presence of osteopontin and osteocalcin, and the absence of bone sialoprotein—indicates that periodontal ligament can be differentiated from other periodontal tissues within the alveolus (i.e., dentin, cementum, and alveolar bone).

**Mesenchymal Stem Cells Acquire Periodontal Ligament Characteristics**

The staining pattern for mesenchymal stem cells was different from that seen for periodontal ligament. Mesenchymal stem cells have a low level of osteocalcin staining in comparison with periodontal ligament and show a flattened, subordinated, or circular morphology at low magnification (compare Fig. 2, panels A and B, with panels C and D, respectively). Co-culture of periodontal ligament with mesenchymal stem cells for 7 days led to an overall change in the mesenchymal stem cell structure, to a more fibroblast-like morphology (Fig. 2E, arrows). Moreover, osteocalcin expression was significantly up-regulated in mesenchymal stem cells following co-culture for 7 and 21 days (Fig. 2I). Osteocalcin staining was localized to the interior of the cells, indicating that this protein was bound to the outside membrane at detectable levels (Fig. 2H, arrows).

Mesenchymal stem cells express bone sialoprotein at high levels, but little bone sialoprotein was detected in periodontal ligament (compare Fig. 3, panels A and B, with panels C and D, respectively). Co-cultures after 7 and 21 days showed that bone sialoprotein expression was reduced (Fig. 3, E and F). Expression of bone sialoprotein in mesenchymal stem cells was significantly reduced after co-culture for 7 days and continued to be reduced for at least 21 days (Fig. 3H). Control samples stained...
Mesenchymal stem cells express lower levels of osteopontin than periodontal ligament (compare Fig. 4, panels A and B, with panels C and D, respectively), and osteopontin staining in mesenchymal stem cells increased by 7 days of co-culture and continued to 21 days (Fig. 4, panels E and F, respectively). Quantitative analysis of the osteopontin staining indicated that this increase was significant (Fig. 4I). Control samples stained negative (Fig. 4G). Bone sialoprotein and osteopontin were localized to the interior of the mesenchymal stem cells in co-cultures similar to osteocalcin (data not shown). Although periodontal ligament expressed BMP-2/4 and collagen III in vitro, we did not observe differences in staining compared with mesenchymal stem cells (data not shown).

Mesenchymal stem cells did not obtain the periodontal marker expression due to the culture conditions, since culturing the mesenchymal stem cells in BioWhittaker's MSCBM medium or the minimal essential medium used for co-culturing did not change the OD of the 3 markers analyzed (osteocalcin [MSC medium, 249 ± 21]; MEM medium, 200 ± 24); bone sialoprotein [MSC medium, 426 ± 20]; MEM medium, 400 ± 41); and osteopontin (MSC medium, 81 ± 11; MEM medium, 78 ± 14).

**DISCUSSION**

These studies tested the capacity of mesenchymal stem cells to differentiate into periodontal ligament cells in vitro. Periodontal ligament cells were identified through morphological characteristics and protein markers, collagen III, osteopontin, BMP-2/4, osteocalcin, and bone sialoprotein. The patterns of expression for these markers were similar in vivo and in vitro, with the exception of detectable amounts of bone sialoprotein found in cultured periodontal ligament. Co-culturing bone marrow-derived mesenchymal stem cells and periodontal ligament led to a significant increase in mesenchymal stem cell expression of osteocalcin and osteopontin and a significant decrease in bone sialoprotein, characteristics of periodontal ligament in vivo. Increased protein expression was observed for osteopontin, osteocalcin, and bone sialoprotein.

![Figure 3. Bone sialoprotein staining of periodontal ligament and mesenchymal stem cells.](image-url)

In panels A, C, E, and F, cell cultures were stained with bone sialoprotein (green) and labeled for the Y chromosome (red dots). In panels B and D, bone sialoprotein staining was completed using DAB (brown). Bone sialoprotein staining of mesenchymal stem cells is shown in panel A (green) and panel B (brown). Periodontal ligament cells express low levels of bone sialoprotein in vitro, as shown in panel C (green) and panel D (brown). (E) Co-cultures of mesenchymal stem cells and periodontal ligament (1:1) after 7 days in vitro. (F) Mesenchymal stem cells (white arrows) express bone sialoprotein after co-culture (1:1) with periodontal ligament for 21 days. Panels A, C, E, and F are images from slides run in the same staining experiment and processed through the same solutions. (G) Negative control showing the DAB staining of periodontal ligament cells incubated with non-immune serum without bone sialoprotein antibody. (H) Histogram showing the mean optical density for mesenchymal stem cells (MSC) and periodontal ligament cells (PDL) and co-cultures after bone sialoprotein fluorescent staining. Optical density values were given as the mean ± SEM. Values were significantly different (P < 0.0001) as shown by ANOVA. Asterisk = P < 0.05, and double asterisk = P < 0.01 as compared with MSC. N ≥ 6. Bars = 50 μm (A, B, F) and 100 μm (C, D, E).
induced mesenchymal stem cells to obtain periodontal-ligament-like characteristics. Importantly, analysis of the data suggests the feasibility of utilizing mesenchymal stem cells in clinical applications for repairing and/or regenerating periodontal ligament tissue.

**Protein Markers in Periodontal Ligament**

The staining pattern we observed for collagen III, osteopontin, osteocalcin, and bone sialoprotein in the periodontal region *in vivo* was consistent with patterns reported previously (Lekic *et al.*, 1996; Ivanovski *et al.*, 2001). Moreover, our results indicate that the *in vivo* staining pattern of the periodontal ligament was similar to the cell staining pattern when cultured *in vitro*. BMP-2/4 and collagen III were expressed at similar levels (data not shown) in both mesenchymal stem cells and periodontal ligament and were not used as markers to differentiate between the two cell types. The BMP-2/4 staining pattern was inconsistent with that reported in previous studies, possibly due to the antibody specificity (Ivanovski *et al.*, 2001). During root development, the pattern of staining for these proteins is likely different, suggesting that regenerative and repair processes are different (D’Errico *et al.*, 1997), supporting the idea that care is required in obtaining donor explant tissue so that selection is from individuals of similar age (e.g., completed oral development in contrast to pediatric tissue).

**Differentiation of Mesenchymal Stem Cells into Periodontal Ligament Fibroblasts**

Significant increases in the optical density of osteocalcin and osteopontin staining and significant decreases in bone sialoprotein staining in mesenchymal stem cells after culture with periodontal ligament fibroblasts for 7 days were detected with little or no observable increase after longer periods. This observation was specific to the effects of co-culturing mesenchymal stem cells with periodontal ligament, since growth of the mesenchymal stem cells in BioWhittaker's MSC/M medium...
or minimal essential medium did not cause a change in marker expression. Moreover, the change in marker expression was not due to excretion of a particular protein from the periodontal ligament and subsequent attachment to the membrane of mesenchymal stem cells, since confocal microscopy showed that the proteins were localized within the cell. Thus, analysis of the data, collectively, suggests that a contact-mediated factor(s) and/or secreted factor(s) induces the process by which mesenchymal stem cells obtain periodontal-ligament-like marker expression.

Collagen III and bone sialoprotein are useful markers in that they aid in differentiating among adult periodontal ligament (high collagen III, low bone sialoprotein expression), gingiva (low collagen III, low bone sialoprotein expression), cementum, dentin, and bone (absent or low collagen III, modest bone sialoprotein expression) (Lekic et al. 1996; Ivanskova et al. 2001). Moreover, osteocalcin and osteopontin are observed in the periodontal ligament, cementum, dentin, and bone, but little or none is observed in the gingiva (data not shown) (Ivanskova et al. 2001). These 4 proteins can form the basis for the identification of mesenchymal stem cell differentiation into various periodontal cell types. For example, if mesenchymal stem cells (modest collagen III, high bone sialoprotein expression) differentiated into gingival fibroblasts, we would observe a decrease in both markers. Thus, observed changes in gene expression of these 4 markers or other potential genes (i.e., cementum attachment protein, dentin sialoprotein) would determine the extent to which mesenchymal stem cells differentiate into the cell types present in the periodontal region.

In conclusion, our results demonstrate mesenchymal stem cells' potency to develop periodontal ligament characteristics and suggest that the cells may have the potential to form other periodontal tissues. Moreover, we describe an in vitro periodontal model in which the genes or molecular pathways leading to these differentiation events can be determined. Studies of mesenchymal stem cell potential to form all the various periodontal ligament tissue types in vitro and in vivo, plus an understanding of this process, can yield highly beneficial clinical applications for repairing and/or regenerating periodontal tissue.

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REFERENCES


