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Production of osteopontin by cultured porcine epithelial cell rests of Malassez

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Background: The epithelial cell rests of Malassez (ERM) are derived from Hertwig's epithelial root sheath (HERS). During development the cells of HERS deposit a variety of molecules on the newly forming root surface. The possibility that ERM retain this potential after root development is completed and secrete bone or cementum-related proteins needs to be investigated. The purpose of this study was to determine the expression of the non-collagenous proteins osteopontin (OPN) and bone sialoprotein (BSP) by cells derived from the epithelial cell rests of porcine periodontium.

Methods: ERM and fibroblasts were cultured from porcine periodontal ligament. The cells were identified and characterized using transmission electron microscopy, immunohistochemistry, western blot analysis of proteins, reverse transcription–polymerase chain reaction and ability to form mineralized nodules in culture. In particular the expression of the mineralized tissue-related proteins, BSP and OPN, was studied.

Results: Cells from porcine periodontal ligaments were successfully cultured; separated and characterized as being of either an epithelial or fibroblastic phenotype. Although the ERM did not form mineralized nodules in culture, they did express a significant amount of mRNA for OPN.

Conclusion: The results from this study provide evidence that ERM express mRNA for at least one bone/cementum-related protein. Whether this function would be consistent with a role for ERM in tissue formation, inflammation and regeneration remains to be established.

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The function and role of the epithelial cell rests of Malassez (ERM) of the periodontal ligament are still largely unknown (1). ERM are derived from Hertwig's epithelial root sheath (HERS), which, during development, deposits a variety of molecules on the dentin surface of the developing root. As the root develops, the HERS disintegrates and the residual cells become the ERM and form an integral component of the normal periodontium. Although the ERM are believed to contribute very significantly to the maintenance of the periodontal attachment, the way in which they do this is still unclear. Whether these cells retain the properties of their parent HERS cells with regards to the ability to secrete proteins of importance for root formation is of interest.

Cementum is usually limited in its location to the surface of tooth roots, and is a unique avascular, non-innervated, mineralized tissue possessing low remodeling potential. It does, however, share many biochemical

properties with other mineralized tissues, particularly bone (2). Compared to bone, cementum is type I collagen predominant (3) and its constituent non-collagenous proteins are similar to bone and include bone sialoprotein (BSP), osteopontin (OPN), osteocalcin, vitronectin and fibronectin (4–7).

The cells of the periodontium are known to express a wide array of cell surface and extracellular matrix molecules. Expression of several bone-related proteins by cultured human gingival and periodontal ligament fibroblasts *in vitro* has been demonstrated by reverse transcription–polymerase chain reaction (RT–PCR) and immunohistochemistry (8–11). OPN and BSP have also been identified in regenerating periodontal ligament (5).

More recently, periodontal ligament cells and cementoblasts from the developing root surface of mice have been successfully isolated and cultured (12–14). From these studies, cementoblasts were found to express transcripts for BSP, OPN, osteocalcin, alkaline phosphatase, osteoblast-specific transcription factor, parathyroid hormone, parathyroid hormone related protein receptor 1 and type I collagen.

However, to date very little is known about the expression of such molecules by ERM. One study has provided some immunohistochemical evidence of OPN expression by HERS cells around mouse mandibular first molars (15). Furthermore HERS cells are thought to deposit a special matrix on the newly formed dentin of developing roots which permits future cementoblasts to attach and commence the formation of the cementum lining of the root surface (16). Therefore, because the ERM are a direct lineage from the HERS, we have hypothesized that the ERM retain the ability of the HERS to secrete a matrix conducive to cementum formation. Two molecules that appear to be of particular importance in this developmental sequence are BSP and OPN (4). Hence, the aim of this study was to determine whether cultured ERM had the capacity to synthesize bone-like matrix molecules such as BSP and OPN.

Materials and methods

Cell culture

Periodontal ligament cells — The method for culturing epithelial and fibroblastic cells from porcine periodontal ligament was as described by Brunette *et al.* (17). Mandibular molars were freshly extracted from 14-week-old pigs obtained from the piggery unit, Gatton campus, University of Queensland in accordance with animal ethics approval (DENT/309/01/ADRF/PHD).

The tissues were immediately washed and stored in phosphate-buffered saline solution (PBS) pH 7.3 and transported to the laboratory. Cultures of periodontal ligament cells were then established from porcine periodontal ligament explants (Fig. 1). A selective trypsinization method was used to separate fibroblasts from epithelial cells obtained from the culture of explants using 0.25% trypsin in citrate saline supplemented with 1% glucose (18). Epithelial and fibroblastic cells from the periodontal ligament were separated and cultured in 75-cm^2 flasks for further experiments. Control ERM cells, to confirm the phenotype of our cultured ERM cells, were kindly provided by Dr Don Brunette, University of British Columbia. These cells were derived from porcine periodontal ligament and have been described in detail previously (17).

Gingival fibroblasts — Gingival fibroblasts were established from a portion of gingiva (8 mm × 8 mm) surgically obtained from an edentulous area between the lower canine and first molar from the same 14-week-old pigs used for collection of periodontal ligament cells as described previously (19). After 5–10 days cells grew from the explants. Once confluence was obtained, the explants were removed and cells were trypsinized and transferred to 75-cm^2 flasks containing Dulbecco's modified Eagle's medium (ICN Biomedicals, Inc., Aurora Ohio, USA) 15% fetal calf serum (Multiser,

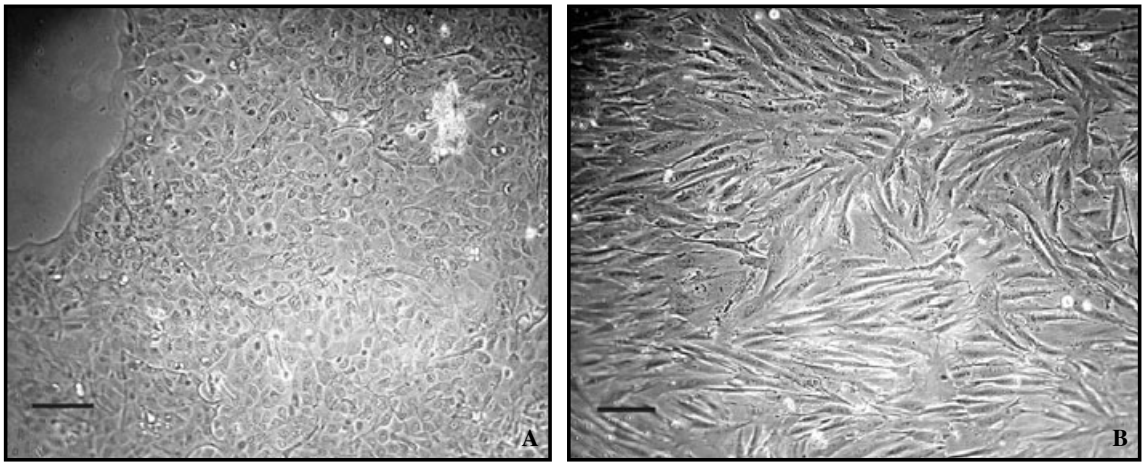

Fig. 1. (A) Photomicrograph of cultured epithelial cells derived from porcine periodontal ligament. Bar = 50 μm. (B) Photomicrograph of cultured fibroblasts derived from porcine periodontal ligament. Bar = 50 μm.

Trace Scientific, Melbourne, Australia), 100 μg/ml penicillin and streptomycin, 50 μg/ml gentamycin (ICN Biomedicals) and 3 μg/ml amphotericin B (ICN Biomedicals) and labeled as porcine gingival fibroblasts.

Alveolar bone cells — Block biopsies of alveolar bone were obtained from the mandibles of 14-week-old pigs to obtain osteoblasts as previously described (20). Briefly, bone biopsies were sectioned into small chips and placed into 25-cm^2 cell culture flasks with alpha modified Eagle's media (ICN Biomedicals) containing 15% fetal calf serum (Multiser, Trace Scientific) and 100 μg/ml penicillin and streptomycin, 50 μg/ml gentamycin (ICN Biomedicals) and 3 μg/ml amphotericin B (ICN Biomedicals). After 2–16 days, cells began to emerge from the biopsies and once they obtained confluence, the cells were transferred, following trypsinization, into a 75-cm^2 tissue culture flask. These cells were labeled as porcine osteoblasts.

Cell characterization

Transmission electron microscopy — Porcine gingival fibroblasts, periodontal ligament fibroblasts and ERM were cultured in either 75-cm^2 or 25-cm^2 cell culture flasks. Upon reaching confluence the media were removed and the cell monolayers were gently washed with PBS. The cultures were then fixed with a primary fixation solution (3% glutaraldehyde in 0.1 M cacodylate buffer) for 2 h at 4°C, and then washed four times with 0.1 M cacodylate buffer for 10 min at 4°C, followed by a secondary fixation in 1% osmium tetra-oxide in potassium ferric-cyanate for 1 h at 4°C. The cells were then washed for 10 min in PBS and followed by two rinses in ultra high purity water at room temperature. The samples were then dehydrated using ascending concentrations of ethanol, 30, 40, 50, 60, 70, 80, 90, and 100%, each time for 10 min. Following dehydration, propylene oxide was applied to the cell layers, which dissolved away the top of the plastic to release the cell layers. The cells were then transferred to a centrifuge tube, rinsed in two washes of propylene oxide, resuspended and lightly centrifuged after each change. Pellets were then immersed in a solvent resin (Quetol/Epon) at concentrations of 2:1, 1:1, 1:2, 100% resin three times and finally polymerized in 100% resin at 37°C overnight and then at 60°C for 3 days. Ultra-thin sections were cut with a diamond knife, then mounted on the grid and stained with 5% uranyl acetate in 50% ethanol for 2 min, followed by a second staining with Reynold's lead stain for 1 min. Sections were then examined by transmission electron microscopy (JEOL JEM 1010, JEOL, Tokyo, Japan).

Immunohistochemistry

Following trypsinization, periodontal ligament fibroblasts, ERM and control cells were plated onto poly-L-lysine slides (Menzel-Glazer, Braunschweig, Germany), left to attach overnight, and then cultured for a further two days. The cells were then fixed with 4% paraformaldehyde 1% glutaraldehyde, washed in PBS and immersed in 3% (v/v) H_2O_2 in PBS for 15 min to eliminate endogenous peroxidase activity. After rinsing in PBS the slides were incubated with 1:10 normal swine serum for 30 min to eliminate non-specific binding. To detect acidic and basic cytokeratins, the cells were then incubated with a 1:1000 dilution of the anti-epithelial keratin-AE1/AE3 antibody (ICN Biomedicals) for 18 h at 4°C. The slides were then rinsed in PBS and incubated with a secondary biotinylated swine-anti-mouse, rabbit goat antibody (Dako LSAB kit, Dako Corporation, Carpinteria, MA, USA) for 15 min. This was followed by incubation with streptavidin peroxidase conjugate for 15 min. Sections were finally treated with 0.5 mg/ml diaminobenzidine in PBS containing 0.15% H_2O_2 for 3 min. Slides were then counterstained with Mayer's haematoxylin, dehydrated and mounted. Negative controls involved the omission of the primary antibody. Staining was assessed under a light microscope (Olympus BX Light Microscope, Olympus Optical, Tokyo, Japan).

Western blot analysis for cytokeratins

Cells cultured in 75-cm^2 flasks were scraped from the flask and homogenized in a lysis buffer containing 25 mM tris HCl (pH 7.4), 1% Triton, 0.6 M KCl, antipan (5 μg/ml), pepstatin (5 μg/ml), 1 mM EDTA, 1 mM EGTA and 1 mM phenylmethylsulphonyl fluoride. All extracts were stored in the extraction buffer at −20°C. In preparation for electrophoresis, 10 μg aliquots of cell lysate from each set of cells were mixed with an equal volume of 2 × loading buffer containing 65 mM dithiothreitol, then heated in 90°C for 5 min. The samples were then loaded onto sodium dodecyl sulfate–polyacrylamide gels comprising a stacking gel of 4% polyacrylamide and a resolving gel of 12.5% polyacrylamide. Electrophoreses was run under 110 V for 4 h.

Separated proteins were subsequently transferred to a nitrocellulose membrane (Hybond ECL, Amersham International, Amersham, Buckinghamshire, UK) by overnight electrophoresis at 30 V. The membranes were incubated in 5% skim milk/PBS for 45 min at room temperature then rinsed twice in Towbin transfer buffer and twice in PBS. The membranes were then incubated with the AE1/AE3 antibody (1:2000 dilution in PBS) and 1% BSA at 4°C overnight. Following washing in Towbin transfer buffer and PBS solutions, the membranes were then incubated with horseradish peroxidase-conjugate secondary antibody (diluted in 1% BSA in PBS 1:2000) at room temperature for 30 min. The membranes were again washed and then developed using ECL solution (Amersham) for 3 min and then exposed to X-ray film.

Mineralization assay – Von Kossa staining

The Von Kossa stain was used to corroborate the calcifying properties of the cultured cells. The cells were transferred to 96-well cell culture plates, and cultured in alpha modified Eagle's media containing dexamethasone sodium phosphate (10 μM), ascorbic acid 2-phosphate (100 μM) and β-glyc-

erophosphate (5×10^{-5} M). The media were changed twice weekly and at day 15 the media were removed from the plates. The cells were then washed twice with PBS prior to addition of 5% silver nitrate. The plates were then exposed to a bright daylight area with aluminium foil placed under the plate for 1 h. The wells were then washed three times with distilled water and allowed to stand in 5% Hypo (sodium thiosulphate solution) for 5 min. The wells were then washed with tap water and rinsed with distilled water. Photographs of the wells were taken to record the areas of calcification.

RNA extraction

Total RNA was isolated from triplicate cultures in 75-cm^2 flasks using acid guanidinium thiocyanate–phenol–chloroform extraction (AB Gene®, Epsom, UK) (21). The cell lysates were transferred immediately to 2-ml Eppendorf tubes and stored at 4°C for 5 min. Following addition of 200 µl of chloroform, each tube was closed tightly and shaken vigorously for 15 s, and allowed to settle at 4°C for 5 min. The homogenates were then centrifuged at 12,000 *g* (4°C) for 15 min. The aqueous phase was transferred to a new Eppendorf tube, and an equal volume of isopropanol was added to precipitate RNA. The precipitates were stored at 4°C for a further 10 min. The samples were then centrifuged at 12,000 *g* (4°C) for 10 min and the precipitated RNA was finally washed twice with 75% ethanol, vortexed and centrifuged at 7500 *g* (4°C) for a further 5 min. The RNA pellet was dried in a vacuum for 5–10 min and then dissolved in 50 µl of ultra-high pure quality water, vortexed for 1 min and incubated at 55–60°C for a further 10–15 min. The RNA concentrations were determined by measuring absorbance at 260 nm in a Gene Quant II RNA/DNA spectroscope (Pharmacia Biotech, Cambridge, UK).

Reverse transcription and polymerase chain reaction

First-strand cDNA was synthesized from 1 µg of total RNA using Oligo dT in a reaction volume of 20 µl reverse transcription mixture containing 1 × RT buffer (Promega, Madison, WI, USA), 0.5 mM dNTPs, 10 mM dithiothreitol, 20 U of RNAse inhibitor and 100 U of M-MLV reverse transcriptase (Promega) at the end of reverse transcription. All samples were heated at 99°C for 5 min to inactivate the enzyme and were stored at −20°C. The nucleotide sequences for each protein were obtained from The National Library of Medicine. The primer sequences for β-2 microglobulin, OPN and BSP were designed using Gene Fisher – Software Support for the Detection of Postulated Genes. The primer sequences and reaction profiles are detailed in Table 1.

Aliquots of 2 µl of the total cDNA were amplified for each PCR in a 20 µl reaction mixture containing 10 pmol of 5′ and 3′ primer for each protein (β-2 microglobulin, OPN or BSP), 10 × PCR buffer (Promega), dCTP, dGTP, dATP, and dTTP each at 0.4 mM, 1 U Taq polymerase. Prior to the addition of 2 µl of the Taq polymerase, the mixture was heated to 94°C for 10 min. For PCR amplifications, denaturation was carried out at 94°C for 60 s, annealing at 57°C for 30 s and extension at 72°C for 80 s.

Reaction products were analyzed and visualized following electrophoresis of 10-µl samples on 2% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide run at 75–80 V for 25–40 min. The relative intensity of each band was determined using image analysis (NIH Image analysis version 1.57, National Institutes of Health, Bethesda, MD, USA). Statistical analysis of the results was carried out using one-way ANOVA analysis with Tukey–Kramer multiple comparisons post-test using InStat View 4.02 software (Abacus Concepts, Inc., Berkley, CA, USA). β−2 microglobulin (β2M) was used as the house keeping gene.

Table 1. Primer sequences and product sizes

β-2 microglobulin	5′: CGAAGGTTCAGGTTTACTC
(312 bp)	3′: ATTCATCCAACCCAGATGC
Osteopontin	5′: ACGACGCTGACCGATCC
(500 bp)	3′: TGTCTTCTTCGCTCTTAGAGTC
Bone sialoprotein	5′: CGACCAAGAGAGTGTCAC
(498 bp)	3′: GCCCATTTCTTGTAGAAGC

Results

Cells from porcine periodontal ligament were successfully cultured, separated and maintained as different cell lines for further experiments. The appearance of the ERM and periodontal ligament fibroblast cultures is shown in Figs 1(A and B).

Transmission electron microscopy

Transmission electron microscopy of epithelial cells obtained from porcine periodontal ligament cultures was characterized by several ultrastructural features. Large numbers of fine cytoplasmic filaments in bundles, tonofilaments, were observed. Desmosomes also characterized these cells and were observed between cell membranes of adjacent cells. Intracellular spaces contained micropodia. The nuclei of the cells were ovoid, contained condensed heterochromatin and nucleoli within the nuclear membrane. The cytoplasm was very dense and contained abundant free ribosomes. The mitochondria were distributed through the cell with lengths of 0.2–0.3 µm. Profiles of rough-surfaced endoplasmic reticulum were only seen occasionally. The Golgi apparatus was not identified in these cells (Figs 2A and B).

In contrast, gingival fibroblasts and periodontal ligament fibroblasts displayed characteristics of cells synthesizing proteins for export such as abundant endoplasmic reticulum studded with ribosomes interspersed with large mitochondria. These fibroblasts did not demonstrate tonofilaments or desmosomes (Fig. 2C).

Immunohistochemistry cytokeratin expression (AE1/AE3 antibody)

Expression of keratins in the cells of the periodontium was demonstrated by immunohistochemistry using the AE1/

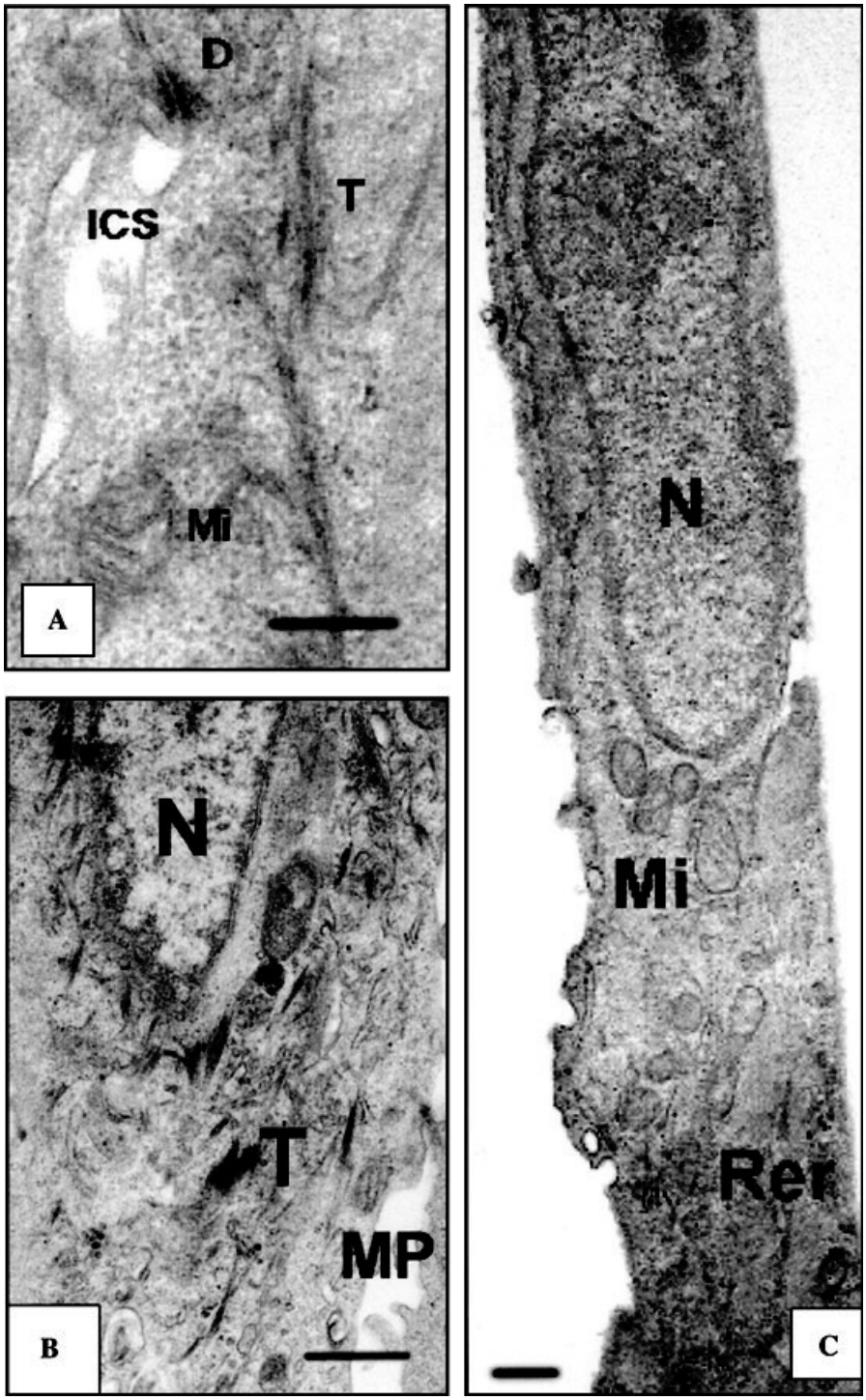

Fig. 2. Electron microscopic appearance of epithelial cells and fibroblasts cultured from porcine periodontal ligament. (A) Two epithelial cells joined by a desmosome (D) The intercellular space (ICS) between the epithelial cells is also apparent. Free ribosomes are abundant. A small mitochondrion is present (Mi). Bar = 500 nm. (B) Portion of the nucleus (N) of an epithelial cell identifiable by tonofilaments (T) and micropodia (MP). Bar = 500 nm. (C) Periodontal ligament fibroblast with characteristic cigar-shaped nucleus (N). The cytoplasm is clear of tonofilaments or desmosomes. Mitochondria (Mi) and profiles of ribosome studded endoplasmic reticulum (Rer) are abundant. Bar = 500 nm.

AE3 antibody. The ERM cells isolated and cultured in this study showed very strong staining and a specific distribution, and also exhibited intercellular connections through the intermediate filaments (Fig. 3A). Cell membranes lacked staining. All cells found in these cultures stained with the AE1/AE3 antibodies, indicating pure cultures of epithelial cells. Periodontal ligament fibroblast cultures were used as negative controls (Fig. 3B).

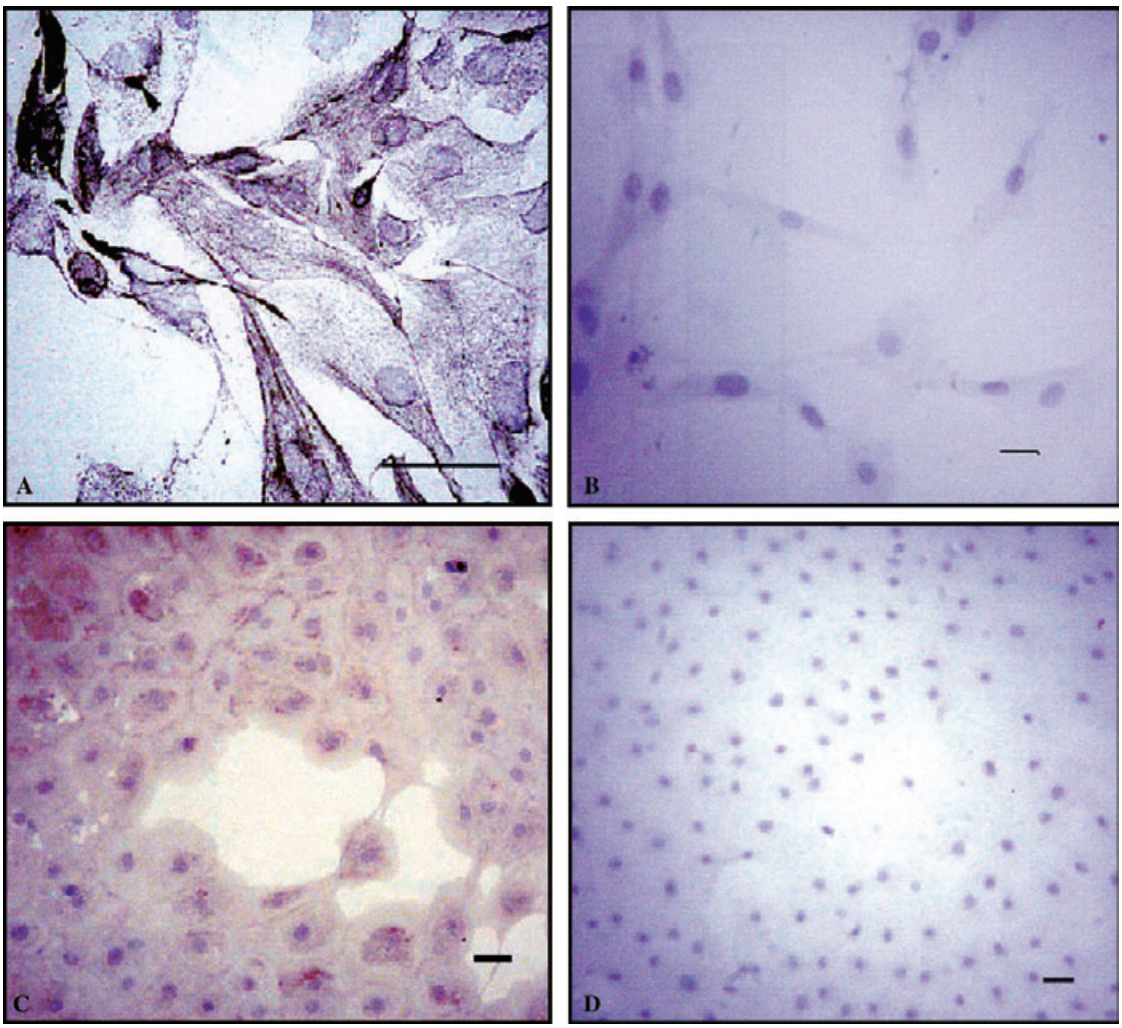

Fig. 3. Immunostaining of cultured epithelial cells and fibroblasts from porcine periodontal ligament for cytokeratins (A) Immunostaining of cultured epithelial cell rests of Malassez (ERM) with AE1/AE3 antibody. Cytoplasmic limits are very well defined despite an absence of stained cell membranes. The patterns of tonofilaments stain brown in cytoplasm and around nuclei. Bar = 100 μm. (B) Immunostaining of cultured periodontal ligament fibroblasts with AE1/AE3 antibody. Bar = 100 μm. (C) Immunostaining of ERM provided by Dr D. Brunette for cytokeratins with AE1/AE3 antibody. The cytoplasm is clearly stained dark brown and the cell boundaries are well delineated. Bar = 100 μm. (D) Negative control immunostaining of ERM provided by Dr D. Brunette. The cell cytoplasm is hard to distinguish but the cell nuclei stain blue with the haematoxylin. Bar = 100 μm.

Control porcine ERM, obtained from Dr Don Brunette, stained strongly in their cytoplasm with AE1/AE3 antibody. The pattern of staining and distribution was consistent with tonofilaments, which comprised both basic and acidic cytokeratins (Fig. 3C). Controls, where the primary antibody was omitted, did not stain for AE1/AE3 (Fig. 3D).

Western blot analysis for cytokeratins

Immunoblot analysis showed several bands from the ERM sample that reacted with the AE1/AE3 antibodies. Most of the protein bands were condensed closely, demonstrated strong intensity and were located within the 40–67 kDa. Control experiments with periodontal ligament fibroblasts demonstrated virtually no staining for proteins within the cytokeratin size limits (Fig. 4).

Von Kossa staining

After culturing in media conducive for mineralization for 14 days, positive deposits were found among the osteoblast cells and sporadically in the periodontal ligament fibroblasts. No Von Kossa staining was detected in the gingival fibroblasts and ERM culture plates (Fig. 5).

Reverse transcription–polymerase chain reaction

The RT–PCR analysis of mRNA extracted from the four sets of cell lines demonstrated significant differences between each of the cell strains for OPN and BSP expression. The agarose gels containing RT–PCR products are presented in Fig. 6 and the relative band intensities compared to β-2

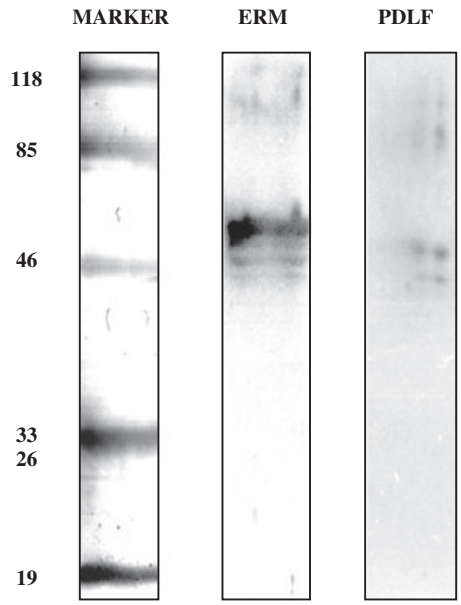

Fig. 4. Western blot for cytokeratins associated with porcine periodontal ligament cells using the AE1/AE3 antibody. Lane 1: standard molecular weight markers; lane 2, epithelial cell rests of Malassez (ERM) cells; lane 3, periodontal ligament fibroblasts (PDLF).

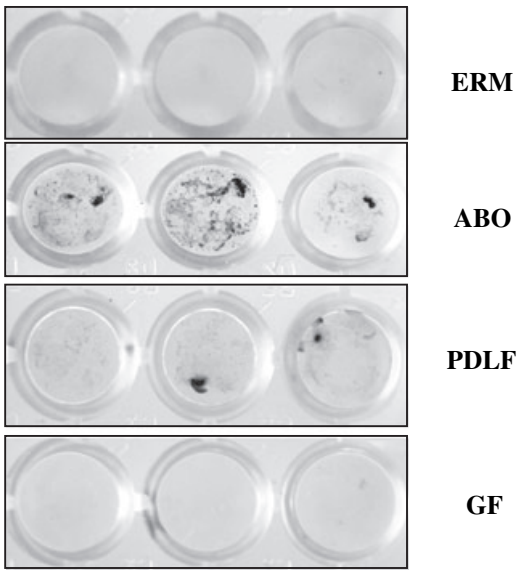

Fig. 5. Von Kossa staining of cultured epithelial cell rests of Malassez (ERM), alveolar bone cells (ABO), periodontal ligament fibroblasts (PDLF) and gingival fibroblasts (GF).

microglobulin are graphically illustrated in Fig. 7.

OPN mRNA expression was variable in all four cell lines. The ERM demonstrated the strongest mRNA detection, whereas the osteoblast cells and periodontal ligament fibroblasts showed a strong mRNA OPN signal but weaker than the ERM cells. The lowest mRNA detection for OPN was seen for the gingival fibroblasts.

Statistical analysis of the OPN mRNA expression showed a significant difference between ERM and the other three periodontal cells ($p < 0.001$). There was no difference of expression of OPN mRNA between osteoblast and periodontal ligament fibroblasts. The detection of OPN mRNA in gingival fibroblasts was significantly lower than the detection in all other samples ($p < 0.001$).

BSP mRNA was weakly detected in osteoblast cells and periodontal ligament fibroblasts. The band intensity for these two cell lines was very similar. BSP mRNA was not detected in gingival fibroblasts and ERM.

Discussion

This study describes ultrastructural, immunohistochemical, western blot analysis and RT–PCR analysis of cells purported to be derived from the ERM of porcine periodontal ligament. These methods were successful in demonstrating the epithelial nature of these cells derived from porcine periodontal ligament.

Electron microscopic evaluation demonstrated the presence of tonofilaments and desmosomes, abundant free ribosomes and little rough-surfaced endoplasmic reticulum. These are all considered to be ultrastructure characteristics of ERM (22–27). Prior studies have described the expression of different cytokeratins by ERM in histological sections using immunohistochemistry or immunofluorescence (27–32). The pattern of immunohistochemical staining for cytokeratins of the cultured ERM in the present study, using AE1/AE3 antibodies, revealed the intermediate filaments were of the tonofilament type. Hence this provides good morphologic correlation with the previously reported ultrastructural histologic findings. This technique has not been used previously on cultured ERM and it establishes that cultured ERM, obtained from the middle third root of the porcine periodontal ligament, express cytokeratins and verifies that these cultures contained cells of an epithelial phenotype. Accordingly, this AE1/AE3 antibody appears to be useful for characterizing cultured ERM cells.

Western blot analyses identified the presence of cytokeratins associated with the cultured ERM in the range of 40–70 kDa. Previous studies have described the use of this technique to study cytokeratin protein expression by a variety of mammalian epithelial cells, including those of human epidermis (33, 34). Unexpectedly, extracts of cultured periodontal ligament

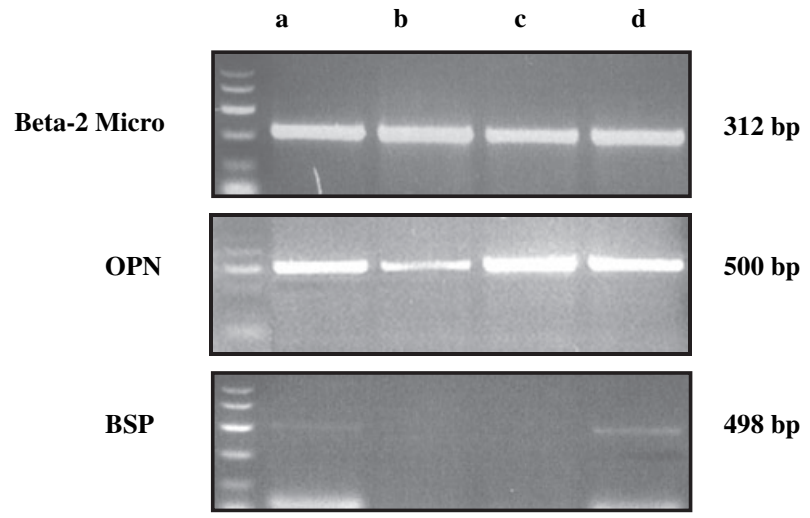

Fig. 6. Reverse transcription–polymerase chain reaction analysis for expression of mRNA for bone sialoprotein (BSP) and osteopontin (OPN). β-2 microglobulin (beta-2 Micro) was used as a standard control. Markers for 1000, 750, 500, 250, 150 and 75 base pairs are shown in the first lane. Lane a, periodontal ligament fibroblasts; lane b, gingival fibroblasts; lane c, epithelial cell rests of Malassez; lane d, alveolar bone cells.

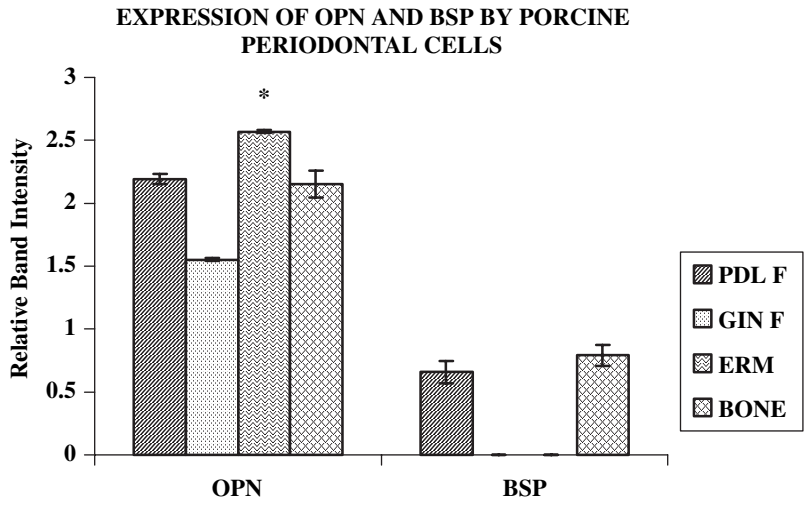

Fig. 7. Graphical representation of relative band intensities standardized against staining for β-2 microglobulin. OPN, osteopontin; BSP, bone sialoprotein; PDL F, periodontal ligament fibroblasts; GIN F, gingival fibroblasts; ERM, epithelial cell rests of Malassez; BONE, alveolar bone cells.

fibroblasts displayed a very weak band for these cytokeratins. This may have resulted in a few residual ERM remaining in these cell cultures derived originally from mixed cultures and subsequently separated by serial trpysinization.

Von Kossa staining of the cultured cells corroborated the mineral-forming capacity of porcine osteoblast and periodontal ligament fibroblasts. Cultures of ERM and gingival fibroblasts did not exhibit this capacity. These results are comparable to previous studies using Von Kossa staining of cultured periodontal ligament cells (35).

This study has demonstrated the expression of OPN mRNA by cultured porcine ERM using a semi-quantitated RT–PCR technique. The signal for OPN mRNA in the ERM was stronger than the expression by periodontal ligament fibroblasts, osteoblast and gingival fibroblasts. BSP mRNA was not expressed by ERM and gingival fibroblasts. Bone and periodontal ligament cells expressed BSP mRNA weakly. The results for periodontal ligament fibroblasts and gingival fibroblasts are comparable with previous studies describing the RT–PCR expression of bone-related proteins in these cells (8–11). More recently, one study has reported the expression of mRNA for some bone-related proteins, including alkaline phosphatase, OPN, bone morphogenetic proteins 2 and 4 in human ERM and periodontal ligament fibroblasts (36).

Three different functions for OPN in calcified tissues have been postulated: regulation of bone cell adhesion, regulation of osteoclast function and regulation of matrix mineralization (37, 38). OPN is also known to participate in cementum formation and has been localized on the root surface during root formation by transmission electron microscopy immunogold techniques (5, 39). It is interesting to note that OPN expression by ERM has been demonstrated in experimental cementum repair (15). In these lesions the ERM were not immunoreactive for OPN at day 3 but were at days 10, 14 and 28. This protein was positively expressed by the ERM situated close to the resorption lacunae. This suggests that ERM might need some sort of stimulation prior to expressing proteins such as OPN, which could be involved in mineralized tissue repair. Such a response has been previously reported for periodontal ligament fibroblasts (40). The high expression of OPN by ERM leads to the speculation of a possible role for these cells in cementum formation. Alternatively, some studies have proposed the transformation of epithelial cells to cementoblasts during root development stages after HERS disruption (41, 42).

In contrast to OPN, BSP mRNA was expressed only by osteoblast cells and periodontal ligament fibroblasts but was not expressed by gingival fibroblasts and ERM. Previous studies have demonstrated the expression of BSP by periodontal ligament cell lines

and cementoblasts isolated from mice (7, 13, 14). These results confirm that BSP is a protein that remains localized to the bone and root surface. In contrast, OPN is noted within the periodontal ligament region of mature tooth (43).

Unfortunately, in this study we were unable to obtain porcine sequences for other bone-related proteins such as osteocalcin, bone morphogenetic proteins 2 and 4, osteonectin and alkaline phosphatase, due to the fact that at the time of carrying out the experiments these sequences had not been described.

In conclusion, using transmission electron microscopy, immunohistochemistry and western blot techniques we have isolated and characterized ERM from porcine periodontal ligament and provided evidence that ERM express mRNA for at least one bone-related protein. Whether such a function enables these cells to participate in tissue repair and regeneration remains to be established.

Acknowledgements

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