Journal of
PERIODONTAL RESEARCH

J Periodont Res 2010; 45: 309–316

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2009.01237.x

# Induction of MMP-2 at the interface between epithelial cells and fibroblasts from human periodontal ligament

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*Shimonishi M, Takahashi I, Terao F, Komatsu M, Kikuchi M. Induction of MMP-2 at the interface between epithelial cells and fibroblasts from human periodontal ligament. J Periodont Res 2010; 45: 309–316. © 2009 John Wiley & Sons A/S*

*Background and Objective:* MMP-2 can degrade type IV collagen and MMP-14 can activate pro MMP-2. The present study was undertaken to examine the expression of MMP-2 and MMP-14 with respect to interaction between the cells of the epithelial rests of Malassez and fibroblasts from human periodontal ligament.

*Material and Methods:* Explants of human periodontal ligament tissues produced outgrowths containing both putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts after incubation in a modified serum-free medium. The distribution and expression of MMP-2 and MMP-14 were analysed using immunohistochemistry, *in situ* hybridization and RT-PCR analysis. The conditioned media and cell extracts were collected for western blot analysis for MMP-2.

*Results:* Putative epithelial rests of Malassez cells at the interface between the cells of the epithelial rests of Malassez and fibroblasts expressed MMP-2 and MMP-14 strongly. However, *in situ* hybridization analysis revealed that human periodontal ligament fibroblasts expressed MMP-2 mRNA while putative epithelial rests of Malassez cells expressed MMP-14 mRNA at the interface. The RT-PCR analysis showed that the expression of MMP-2 mRNA was significantly higher when putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts were cultured together than when cultured alone. Western blot analysis showed that the active form of MMP-2 was detected at higher levels in the conditioned medium of the co-cultured cells.

*Conclusion:* These findings indicate that putative epithelial rests of Malassez cells stimulate the production of MMP-2 in human periodontal ligament fibroblasts. Up-regulated proMMP-2 bound by MMP-14 expressed in epithelial rests of Malassez cells can degrade matrix molecules, such as type IV collagen, in the basal membrane between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts.

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Key words: *in vitro* model; MMP-2; MMP-14; cultured epithelial rests of Malassez

Accepted for publication May 4, 2009

Normal epithelial cells are separated from underlying mesenchyme by a specialized layer of extracellular matrix (ECM) termed the basement membrane, which consists of type IV collagen and laminin, forming the main structural element. Similarly, the epithelial rests of Malassez are surrounded by a continuous basement membrane (1,2). We demonstrated that synthesis of type IV collagen and laminin can be induced by direct interaction at the interface between the epithelial rests of Malassez cells and fibroblasts from human periodontal ligament (3). Some reports have suggested that the epithelial rests of Malassez play a role in maintenance of the

periodontal space (4–9). However, much remains to be learned about various aspects of epithelial–mesenchymal interactions and their role in homeostasis of the periodontium. Specifically, epithelial–mesenchymal interactions participate in the process of tissue remodelling, repair and regeneration, encompassing both the construction and destruction of the components of the ECM. Degradation of the ECM may involve as many as four apparently distinct pathways: osteoclastic, plasminogen-dependent, phagocytic and matrix metalloproteinase (MMP) pathways (10). It is important to focus specifically on MMP pathways in the maintenance of the periodontal space because of their intrinsic relationship with chronic periodontal disease, chronic periapical cyst formation and the healthy periodontal ligament.

Matrix metalloproteinases can initiate tissue remodelling by degradation of ECM. Matrix metalloproteinase-2, in particular, has been shown to play an important role in turnover or degradation of connective tissue, in both physiological and pathological conditions (11,12). Matrix metalloproteinase-2 can degrade type IV collagen, the major protein found in the basement membrane. The activated MMP-14, membrane-type-1 matrix metalloproteinase (MT1-MMP), binds the tissue inhibitor of metalloproteinases (TIMP)-2 by its N-terminal inhibitory domain. The C-terminal domain of the bound TIMP-2 acts as a receptor for binding the C-terminal haemopexin domain of proMMP-2, and MMP-14 can activate proMMP-2 (13–16).

According to Salo *et al*., the expression of MMP-2 mRNA is localized in the connective tissue fibroblasts and endothelial cells of human oral mucosa, and mucosal epithelium is negative for MMP-2 mRNA expression during wound healing (17). However, cultured epithelial cells from porcine rests of Malassez can produce MMP-2 (18–20). Since little is known about the induction of MMP-2 from epithelial–mesenchymal interactions *in vitro*, the objective of the present study was to determine whether the epithelial rests of Malassez cells and fibroblasts from human periodontal ligaments at the interface *in vitro* have the ability to regulate MMP-2 and MMP-14, thus clarifying their role in maintenance of the periodontal ligament.

## Material and methods

### Cell culture

Freshly extracted third molars from 30 patients between 17 and 28 years of age were obtained from the Oral Surgery Department, Tohoku University Graduate School of Dentistry. Informed consent was obtained from the patients prior to extractions. The human ethics board of Tohoku University Graduate School of Dentistry specifically granted permission for our project to work with human subjects. After washing the teeth several times with α-minimal essential medium (α-MEM; (Cosmo Bio Co. Ltd, Tokyo, Japan) supplemented with 10% fetal bovine serum and antibiotics (60 μg/mL kanamycin, 20 units/mL penicillin G and 10 μg/mL amphotericin B), some human periodontal ligament explants attached to the mid-third of each root were carefully removed from the root with a scalpel. Explants plated onto 35 mm culture dishes, supplemented with α-MEM, produced outgrowths composed primarily of fibroblasts. One week later they were cultured in a modified serum-free 3:1 (vol/vol) MCDB153 medium (Sigma Chemical Co., St Louis, MO, USA), supplemented with 5 μg/mL insulin (Sigma Chemical Co.), 0.5 μg/mL hydrocortisone (Sigma Chemical Co.), 10 μg/mL transferrin (Sigma Chemical Co.), 14.1 μg/mL phosphorylethanolamine (Sigma Chemical Co.), 10 ng/mL epidermal growth factor (Sigma Chemical Co.), α-MEM including 40 μg/mL bovine pituitary extract (Kyokuto, Tokyo, Japan) and antibiotics (60 μg/mL of kanamycin, 20 units/mL of penicillin G, 10 μg/mL of fungisone) (3). Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$. This procedure resulted in outgrowths of epithelial cells as well as fibroblasts and has been reported previously (3).

From cultures that contained both cell types, differential adhesion to the surface in the presence of 0.075 mg/mL protease solution (Sigma Chemical Co.) produced cultures predominantly of one cell type, either fibroblasts (5–10 min) or epithelial cells (15–20 min) (21). Putative epithelial rests of Malassez cells cultured alone (PE cells) and periodontal ligament fibroblasts cultured alone (PF cells) were used as controls.

### Immunohistochemistry

The cells were fixed in 4% paraformaldehyde at room temperature for 10 min. hydrogen peroxide ($H_2O_2$; 3%) was used to inhibit endogenous peroxidase. Then the cells were incubated with 5% normal goat serum for 30 min to block non-specific binding. To confirm that they were epithelial cells, they were reacted at 4°C overnight with monoclonal mouse anti-human cytokeratin AE1/AE3 (1:50 dilution; Dako, Carpintaria, CA, USA). The cells were also incubated at 4°C overnight with primary antibodies to monoclonal mouse anti-human type IV collagen (1:500 dilution; Sigma Chemical Co.), rabbit polyclonal anti-human MMP-2 (1:500 dilution; Chemicon International Inc., CA, USA) and rabbit polyclonal anti-human MMP-14 (1:100 dilution; Chemicon International Inc.). After rinsing the cells in phosphate-buffered saline (PBS), they were incubated with biotinylated immunoglobulin at room temperature for 30 min and stained with the avidin–biotinylated peroxidase complex (ABC) method, using an ExtrAvidin® peroxidase staining kit (Sigma Chemical Co.) and AEC (3-amino-9-ethylcarbazole) chromogen kit (Sigma Chemical Co.). Cells were counterstained with Mayer's haematoxylin solution. For control staining, PBS was used instead of primary antibody and rabbit serum and also instead of primary antiserum.

### *In situ* hybridization

The oligonucleotide probes used for *in situ* hybridization were synthesized by Nihon Gene Research Laboratories

*Table 1.* Oligonucleotide probes

| Oligonucleotide-name | Sequence (5′ → 3′) | mer | Label | References |
|---|---|---|---|---|
| Amelogenin | CAT GGG TTC GTA ACC ATA GGA AGG | 24 | 3′ Biotin | (22) |
| Type IV collagen α-1 | TCC AGG GTA GCC CCT CTC TCC TTT TTC TCC CAA AGG TCC TGT GCC | 45 | 3′ Biotin | (23) |
| MMP-2 | CAG GTG ACA ACC ACC CTT GA | 20 | 3′ Biotin | (24) |
| MMP-14 | ACG AGT GGT AGA GTC ACT CC | 20 | 3′ Biotin | (25) |

Inc., Sendai, Japan. The sequences are shown in Table 1. A biotin label was added at the 3′ end. A computer-assisted search (GenBank) of the antisense sequences as well as the sense sequences revealed no significant homology with any known sequences other those of the amelogenin, the type IV collagen α-1, the MMP-2 and the MMP-14 gene.

*In situ* hybridization was carried out using the *In Situ* Hybridization Detection Kit for Biotin Labeled Probes (Sigma Chemical Co.). Cells were fixed in 4% paraformaldehyde at room temperature for 10 min, and then immersed in PBS with RNase inhibitor at room temperature. Hydrogen peroxide (3%) was used to inhibit endogenous peroxidase. The specimens were hybridized overnight in a humid incubation chamber at 37°C with biotin-labelled probes in the hybridization solution. After washing in PBS, the specimens were reacted with blocking solution (5% BSA, 500 μg/mL normal sheep immunoglobulin G, 100 μg/mL salmon testicular DNA and 100 μg/mL yeast tRNA in PBS) at room temperature for 15 min. Then they were incubated with ExtrAvidin® peroxidase solution (Sigma Chemical Co.) at 37°C for 20 min. After washing three times in PBS, the sites of peroxidase were visualized using a solution containing 3,3′-diaminobenzidine and $H_2O_2$ and counterstained with Mayer's haematoxylin solution. Sense oligonucleotide probes were used for control staining.

### Reverse transcription-polymerase chain reaction (RT-PCR)

The interface area cells (IEF cells), which are present between putative epithelial rests of Malassez cells and periodontal ligament fibroblasts, were obtained using silicone cylinders. After aspirating the medium and washing twice with PBS, 5 mm diameter silicone cylinders, smeared on the bottom with white petrolatum, were placed on the dishes. Fifty microlitres Buffer RLT, containing guanidine thiocyanate and β-mercaptoethanol (Qiagen Pty Ltd, Doncaster, Victoria, Australia) was added to the cylinders. The cells lysed with Buffer RLT were collected in microcentrifuge tubes. This procedure has been reported previously (26). The PE and PF cells were used as controls. Using an RNeasy® Mini Kit (Qiagen Pty Ltd), total cellular RNA was isolated from cultured cells based on the manufacturer's instructions. Fifty nanograms per microlitre total RNA was used as a template for RT-PCR. Synthesis of cDNA was performed using total RNA primed with random primers (Invitrogen Corp., Carlsbad, CA, USA). Using a Programmable Thermal Controller MyCycler (Bio-Rad Laboratories, Hercules, CA, USA), cDNA obtained in the synthesis reaction was amplified directly using PCR with Taq DNA polymerase (Invitrogen Corp.), according to the manufacturer's instructions. To quantify the expression of MMP-2 and MMP-14, semi-quantitative RT-PCR analysis relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed. Amplimers designed for these four molecules and related information are provided in Table 2. The PCR products were electrophoresed, and digital images were obtained and analysed using IMAGE J (NIH image) software (National Institutes of Health, Bethesda, MD, USA). The consistency of the data was confirmed by three independent experiments. The results were analysed statistically using the Bonferroni/Dunn test, and $p$-values $< 0.05$ were considered significant.

### Western blot analysis for MMP-2

Western blot analysis was performed to examine the level of MMP-2 in the conditioned media from the co-culture, PE cells and PF cells, as well as to examine the level of MMP-2 in the extracts of the co-culture cells, PE cells and PF cells. The conditioned media were collected and concentrated with the Ultrafree-MC PBQK Centrifugal Filter Unit (Millipore, Bedford, MA, USA). Cells were extracted with a buffer containing 50 mM Tris base-HCl (pH 8.0), 150 mM NaCl, 0.5% Triton-X-100, 1 μg/mL leupeptin, 5 μg/mL aprotinin, 0.5 mM phenylmethlsulphonylfluoride (PMSF), 1 mM EDTA, 1 mM EGTA, 1 mM $Na_3VO_4$, 1 mM NaF and 10 mM sodium pyrophosphate. The concentrations of the

*Table 2.* Primer pairs used for RT-PCR amplification

| Gene (fragment) | Primer sequence | Denaturation/annealing/extension (°C) | Cycle | References |
|---|---|---|---|---|
| MMP-2 (173 bp) | 5′-ATGACAGCTGCACCACTGAG-3′<br>5′-ATFTGTTGCCCAGGAAAGTG-3′ | 95/56/72 | 40 | (24) |
| MMP-14 (171 bp) | 5′-CAGAGAAGGCACACAAACGA-3′<br>5′-CACTGGTGAGACAGGCTTGA-3′ | 95/58/72 | 40 | (25) |
| GAPDH (485 bp) | 5′-TGTTTGTGATGGGTGTGAA-3′<br>5′-ATGGGAGTTGATGTTGAAG-3′ | 95/58/72 | 40 | (27) |

total protein in the samples were determined with the Sunrise™ absorbance microplate reader (Tecan, Männedorf, Switzerland), using a Pierce BCA kit (Pierce, Rockford, IL, USA). Samples with equal amounts (10 μg) of total protein (0.5 μg/μL) were mixed with Laemmli sample buffer (Bio-Rad Laboratories) and then denatured by 95°C heat block bath. All samples were subjected to electrophoresis in reducing conditions on a 10% sodium dodecyl sulphate polyacrylamide gel at 500 V and then transferred onto a nitrocellurose membrane at 400 mA for 50 min. The membranes were incubated overnight at 4°C with rabbit polyclonal anti-human MMP-2 (1:1000 dilution; Chemicon International Inc.). The membranes were subsequently incubated with secondary horseradish peroxidase-conjugated immunoglobulin at room temperature for 30 min. Then the bands were amplified using the GenPoint™ Tyramide Signal Amplification System (Dako Cytomation Inc., Carpinteria, CA, USA). The sites of peroxidase were visualized using a solution containing 3,3′-diaminobenzidine and $H_2O_2$.

## Results

### Immunohistochemistry

Putative epithelial rests of Malassez cells stained positive for broad-spectrum antibodies to cytokeratins (AE1/AE3), indicating their epithelial origin, while periodontal ligament fibroblasts did not show cytokeratin expression at the interface in the same dishes (Fig.1A). The intensity of immunostaining for type IV collagen in periodontal ligament fibroblasts immediately adjacent to putative epithelial rests of Malassez cells was markedly high. Also, putative epithelial rests of Malassez cells stained positively (Fig. 1B). Intense immunoreactivity for MMP-2 and MMP-14 was observed in putative epithelial rests of Malassez cells at the interface (Fig. 2A,D). Matrix metalloproteinase-2 proteins were detected weakly in PE cells and PF cells (Fig. 2B,C), but MMP-14 was detected in PE cells and PF cells (Fig. 2E,F).

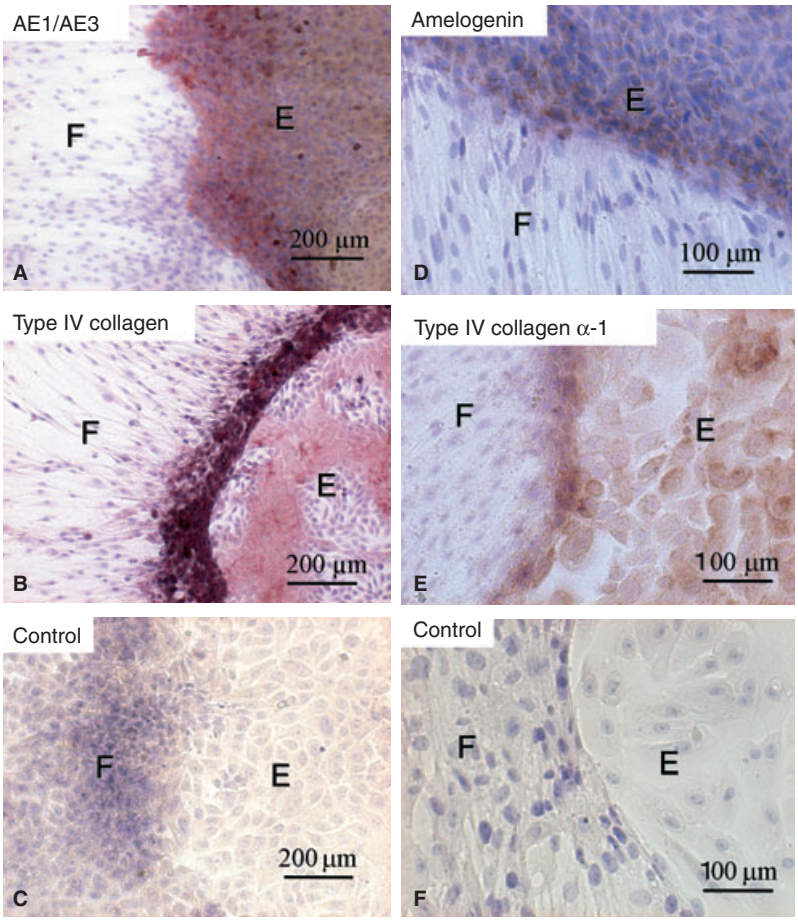


*Fig. 1.* (A) Photomicrograph showing intense immunoreactivity for cytokeratin AE1/AE3 in putative epithelial rests of Malassez cells. (B) Photomicrograph showing intense immunoreactivity for type IV collagen between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts. (C) Photomicrograph showing negative control immunostaining in putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts. (D) Photomicrograph showing intense immunoreactivity for amelogenin mRNA in putative epithelial rests of Malassez cells. (E) Photomicrograph showing intense immunoreactivity for type IV collagen α-1 mRNA between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts. (F) Photomicrograph showing the negative control hybridized *in situ* with biotin-labelled sense oligo-DNA probes for type IV collagen α-1 mRNA in putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts. Abbreviations: E, putative epithelial rests of Malassez cells; and F, human periodontal ligament fibroblasts.

### *In situ* hybridization to localize mRNA for amelogenin, type IV collagen, MMP-2 and MMP-14

To localize the mRNA expression of amelogenin, type IV collagen, MMP-2 and MMP-14, the cells were hybridized *in situ* with biotin-labelled antisense oligo-DNA probes. Putative epithelial rests of Malassez cells showed higher positive signals for amelogenin mRNA, but an amelogenin mRNA signal was not detected in periodontal ligament fibroblasts (Fig. 1D). These results support the hypothesis that putative epithelial rests of Malassez cells are different from periodontal ligament fibroblasts and are of odontogenic epithelial origin. Periodontal ligament fibroblasts immediately adjacent to putative epithelial rests of Malassez cells showed stronger positive signals for type IV collagen α-1 mRNA than other fibroblasts further away from putative epithelial rests of Malassez cells. Moreover, type IV

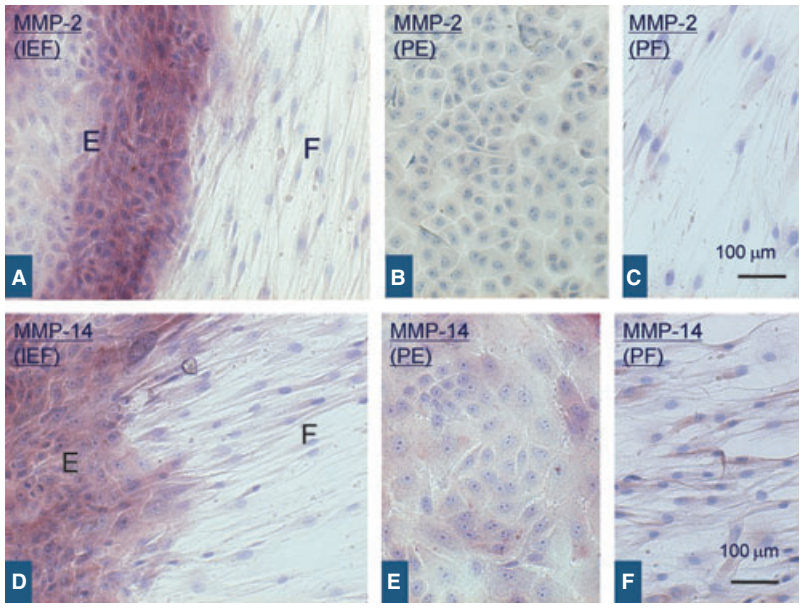


*Fig. 2.* (A–C) Photomicrographs showing strong immunoreactivity for MMP-2 in the epithelial rests of Malassez cells at the interface. MMP-2 proteins were not detected in PE cells or PF cells. (D–F) Photomicrographs showing strong immunoreactivity for MMP-14 in putative epithelial rests of Malassez cells at the interface, PE cells and PF cells. Abbreviations: IEF, interface area cells, which include both putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts at the interface; PE, putative epithelial rests of Malassez cells cultured alone; and PF, periodontal ligament fibroblasts cultured alone.

collagen α-1 mRNA was detected positively in putative epithelial rests of Malassez cells (Fig. 1E). Weak immunoreactivity for MMP-2 mRNA was observed in periodontal ligament fibroblasts at the interface, but there was no immunoreactivity for MMP-2 mRNA in PE and PF cells (Fig. 3A–C). Immunoreactivity for MMP-14 mRNA reflected the immunohistochemical data. Intense immunoreactivity for MMP-14 mRNA was observed in putative epithelial rests of Malassez cells at the interface, PE cells and PF cells (Fig. 3D–F).

### Reverse transcriptase-polymerase chain reaction

Semi-quantitative RT-PCR analysis was used to investigate expressions of two genes (mRNA of MMP-2 and MMP-14) in three cell populations: IEF cells, PE cells and PF cells (Fig. 4). Their relative intensities compared with GAPDH are illustrated graphically in Fig. 5. The expression of mRNA for MMP-2 was significantly higher in IEF cells ($p < 0.05$) than in the other cell populations, but there were no significant differences in the relative expression of mRNA for MMP-14 among the three cell populations.

### Western blot analysis for MMP-2

To identify the bands of MMP-2, we monitored the activation of MMP-2 with western blot analysis in the conditioned media and the cell extracts from all samples: the co-culture, PE cells and PF cells. Western blot analysis showed the three bands respectively, confirming that these three bands were the pro-form, the partially active form and the active form of MMP-2 (Fig. 6). The pro-form and the partially active form of MMP-2 were more prevalent in the cell extracts of the co-cultured cells and PF cells than in the cell extracts of PE cells. Also, the active form of MMP-2 was detected at a higher concentration in the conditioned media from the co-cultured cells and PF cells than in the conditioned medium from PE cells.

## Discussion

It has been suggested that epithelial rests of Malassez may protect the root surface from resorption and prevent ankylosis, thus helping to maintain the integrity of the periodontal ligament. Some studies postulate that proliferating epithelial rests of Malassez cells

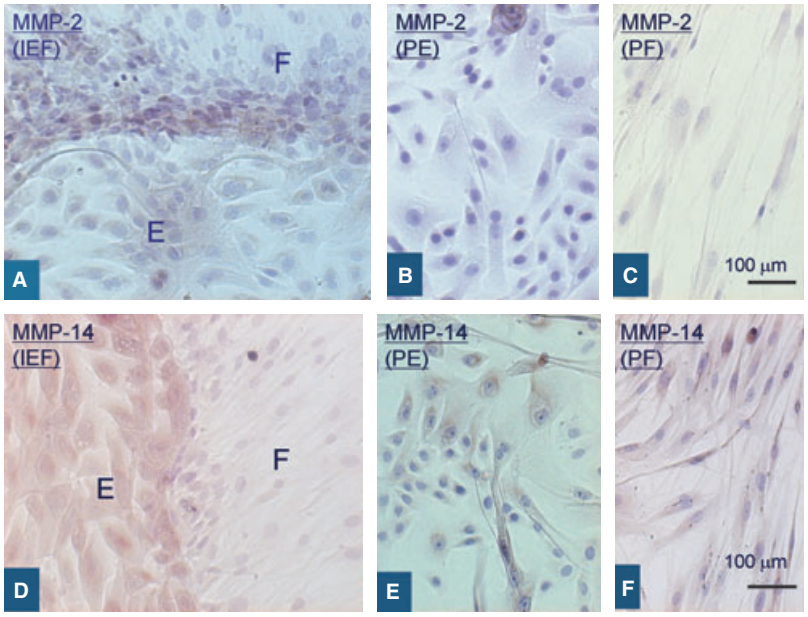


*Fig. 3.* (A–C) Photomicrographs showing immunoreactivity for MMP-2 mRNA in human periodontal fibroblasts at the interface but no immunoreactivity for MMP-2 mRNA in PE cells or PF cells. (D–F) Photomicrographs showing strong immunoreactivity for MMP-14 mRNA in putative epithelial rests of Malassez cells at the interface, PE cells and PF cells. Abbreviations as in legend to Fig. 2.

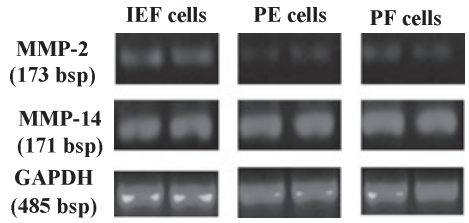


*Fig. 4*. Reverse transcription-polymerase chain reaction analysis of the mRNA expression of MMP-2 and MMP-14 in cultures of IEF cells, PE cells and PF cells. Reverse transcription-polymerase chain reaction products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IEF, interface area cells, which include both putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts at the interface; PE, putative epithelial rests of Malassez cells cultured alone; and PF, human periodontal ligament fibroblasts cultured alone.

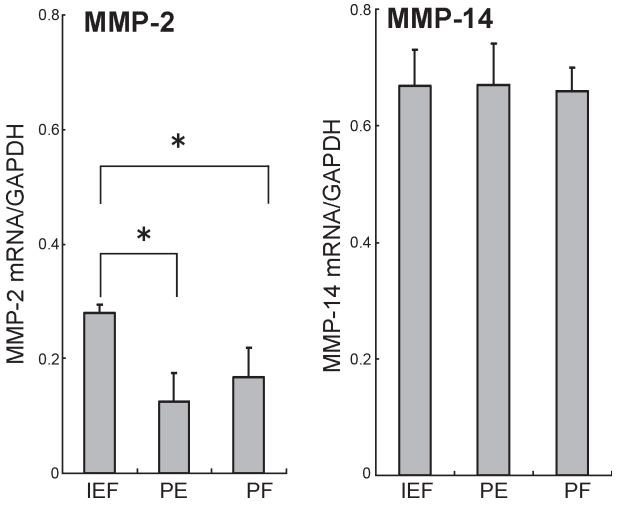


*Fig. 5*. Graphical representation of relative band intensities standardized by GAPDH (means ± SD, $n = 3$; $*p < 0.05$). Abbreviations as in legend to Fig. 4.

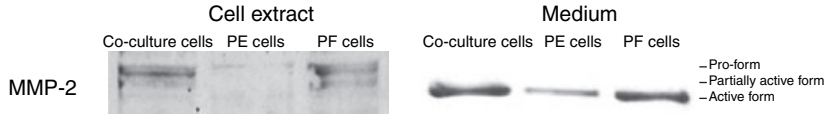


*Fig. 6*. Western blot analysis for MMP-2 in the conditioned media and cell extracts from co-cultured cells, PE cells and PF cells. Abbreviations: PE, putative epithelial rests of Malassez cells cultured alone; and PF, human periodontal ligament fibroblasts cultured alone.

may play a role in the repair of orthodontic root resorption and reconstitution of the periodontium following tooth movement (8,9,28). Rincon *et al*. suggest that epithelial rests of Malassez are crucial for successful and predictable periodontal regeneration (29,30). Since epithelial rests of Malassez are associated with maintenance of the periodontal space, they may be crucial for the secretion and degradation of matrix molecules in the basement membrane. In fact, in our previous study, the synthesis of type IV collagen and laminin, including the basement membrane, was induced by their direct interaction (3). In the present study, as a marker of basement membrane integrity, periodontal ligament fibroblasts immediately adjacent to putative epithelial rests of Malassez cells showed stronger positive signals for type IV collagen and type IV collagen α-1 mRNA than other fibroblasts further away from putative epithelial rests of Malassez cells. Also, type IV collagen was detectable in putative epithelial rests of Malassez cells. The basement membrane is thought to be the prerequisite for epithelial cell adhesion, migration, differentiation and growth. Therefore, type IV collagen might be mainly required both for the reconstruction of the basement membrane at the interface and for the migration of putative epithelial rests of Malassez cells. In fact, putative epithelial rests of Malassez cells grew out and migrated towards periodontal ligament fibroblasts at the interface. In this way, our *in vitro* co-culture system may provide a simple model to investigate epithelial–mesenchymal interaction, whereas an *in vivo* wound healing model contains various other complex factors (10).

Matrix metalloproteinase-2 has been shown to play an important role in turnover or degradation of soft connective tissue. It can degrade type IV collagen, the major ECM protein found in the basement membrane. It has been proposed that MMP-2 activation requires the function of MMP-14. Activated MMP-14 binds TIMP-2 by its N-terminal inhibitory domain. The C-terminal domain of the bound TIMP-2 acts as a receptor for binding the C-terminal haemopexin domain of proMMP-2, and MMP-14 can activate proMMP-2 (13–16). The IEF cells had a significantly higher expression of MMP-2 mRNA than the PE and PF cells. Immunohistochemical data at the interface indicated that MMP-2 was detectable in putative epithelial rests of Malassez cells, while *in situ* hybridization data indicated that MMP-2 mRNA was detectable in periodontal ligament fibroblasts. In contrast, there were no significant differences in MMP-14 mRNA expression among the three cell populations, while hybridization signals of MMP-14 mRNA were stronger than those of MMP-2 mRNA in IEF cells. Immunohistochemical and *in situ* hybridization data indicated that MMP-14 was detectable in epithelial rests of Malassez cells at the interface. These findings

suggest that production of proMMP-2 by the up-regulated MMP-2 gene could bind to and be activated by MMP-14 and degrade type IV collagen at the interface.

In addition, western blot analysis revealed that the pro- form and the partially active form of MMP-2 were more prevalent in the cell extracts of the co-cultured cells and PF cells than in the cell extracts of PE cells. Also, the active form of MMP-2 was detected at higher levels in the conditioned media from the co-cultured cells and PF cells than in the conditioned medium from PE cells. These results support the hypothesis that the pro- form and the partially active form of MMP-2 could bind to MMP-14 in the cell membrane and that the active form of MMP-2 was released in the culture medium after the activation of the pro- form of MMP-2 by MMP-14. Higher levels of MMP-2 were detected in both the conditioned media and the cell extracts of the co-cultured cells as well as the PF cells. However, the co-cultured cells could include putative epithelial rests of Malassez cells, which expressed a lower level of MMP-2. In combination with the RT-PCR results, it seems that MMP-2 could be induced at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts.

Salo *et al*. demonstrated by *in situ* hybridization that fibroblasts of oral resting and healing mucosa are positive for MMP-2 mRNA expression, while the signal of MMP-2 mRNA was absent in the epithelium during *in vivo* wound healing (17). Also, it was demonstrated by zymographic analysis that cultured human oral keratinocytes expressed small amounts of MMP-2 (17). Some reports have shown that cultured epithelial cells produce MMP-2 and MMP-9 (18,19). We found that when putative epithelial rests of Malassez cells are cultured alone, a weak expression of MMP-2 mRNA can be detected in RT-PCR. *In situ* hybridization data showed that weak hybridization signals for MMP-2 mRNA were detected in periodontal ligament fibroblasts at the interface. Okada *et al*. found that during day 3 of wound healing, MMP-14 mRNA can be detected in the superficial dermis adjacent to the proliferating epithelial cell layer, although the expression levels were higher for MMP-2 than for MMP-14 (16). We also observed that intense hybridization signals for MMP-14 mRNA can be detected in putative epithelial rests of Malassez cells at the interface. However, the expression for MMP-14 in our study was the opposite of theirs. These findings suggest that our culture conditions are similar but not identical to *in vivo* conditions during wound healing.

Members of the SIBLING (Small, Integrin-Binding LIgand, N-linked Glycoprotein) family contain the integrin binding tripeptide, Arg-Gly-Asp (RGD), as well as several conserved phosphorylation and N-glycosylation sites (31–33). The SIBLING family can bind proMMPs specifically and activate MMPs. Normally, bone sialoprotein is produced by osteoblasts, osteoclasts, osteocytes, hypertrophic chondrocytes (34) and periodontal ligament fibroblasts, and through its RGD sequence, binds to the integrin, $\alpha_v\beta_3$. Bone sialoprotein is one of the members of the SIBLING family and binds specifically to proMMP-2 and active MMP-2. Bone sialoprotein has been shown to enhance cell invasion *in vitro* by bridging MMP-2 to the cell surface via the $\alpha_v\beta_3$ integrin (35). We have previously demonstrated that bone sialoprotein and its mRNA are expressed to a significant degree at the interface between putative epithelial rests of Malassez cells and fibroblasts from human periodontal ligaments (26). Bone sialoprotein may also activate up-regulated proMMP-2 at the interface. Korostoff *et al*. demonstrated that active MMP-2 and a 40 kDa serine protease were present in chronic adult periodontitis (36). Chronic inflammatory diseases, such as periodontal disease and chronic periapical cyst formation, induced extensive tissue destruction, including that of the adjacent alveolar bone around the tooth. Proliferation of the epithelial rests of Malassez has been implicated in developmental periodontal disease and cyst formation. It may be necessary to facilitate the activation of MMP-2 for the effect of bone sialoprotein produced at the interface between the epithelial rests of Malassez cells and fibroblasts from human periodontal ligaments in inflammatory conditions. While it is accepted that the host response to bacterial products is the major cause of pathogenesis (36–38), there was no influence of bacterial factors in this culture system (present study). Further examinations are required for the confirmation of the effect of bacterial factors on the activation of MMP-2.

In conclusion, these findings indicate that epithelial–mesenchymal interactions stimulate the production of proMMP-2 in periodontal ligament fibroblasts. Up-regulated MMP-2 bound by MMP-14 expressed in putative epithelial rests of Malassez cells can degrade matrix molecules, such as type IV collagen, in the basal membrane between putative epithelial rests of Malassez cells and periodontal ligament fibroblasts. We believe that epithelial–mesenchymal interactions are intimately intertwined with maintenance and remodelling of the periodontal ligament, and as such make a dynamic contribution to homeostasis of the periodontium.

## Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (C) (no. 19592167) from the Japan Society for the Promotion of Science, Japan.

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