Journal of
PERIODONTAL RESEARCH

J Periodont Res 2010; 45: 353–360

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2009.01244.x

Gingival crevicular fluid can degrade Emdogain and inhibit Emdogain-induced proliferation of periodontal ligament fibroblasts

M. Laaksonen[1], T. Salo[2], S. Vardar-Sengul[3,4], G. Atilla[4], B. Han Saygan[4], J. P. Simmer[5], H. Baylas[4], T. Sorsa[1]

[1]Department of Oral and Maxillofacial Diseases, Institute of Dentistry, Helsinki University Central Hospital, University of Helsinki, Helsinki, Finland, [2]Department of Diagnostics and Oral Medicine, Institute of Dentistry, Oulu University Central Hospital, University of Oulu, Oulu, Finland, [3]Department of Periodontology and Oral Biology, Goldman School of Dental Medicine, Boston University, Boston, MA, USA, [4]Department of Periodontology, School of Dentistry, Ege University, Izmir, Turkey and [5]Deparment of Biologic and Materials Sciences, University of Michigan Dental Research Laboratory, Ann Arbor, MI, USA

Laaksonen M, Salo T, Vardar-Sengul S, Atilla G, Han Saygan B, Simmer JP, Baylas H, Sorsa T. Gingival crevicular fluid can degrade Emdogain and inhibit Emdogain-induced proliferation of periodontal ligament fibroblasts. J Periodont Res 2010; 45: 353–360. © 2009 The Authors. Journal compilation © 2009 Blackwell Munksgaard

Background and Objective: Emdogain® (EMD), consisting mostly of amelogenin, is used in periodontal therapy to regenerate lost connective tissue. Emdogain is applied onto periodontally affected root surfaces, where it becomes exposed to proteolytic enzymes. In this study, we aimed to find out whether gingival crevicular fluid or matrix metalloproteinases (MMPs) could degrade EMD, and whether this degradation has consequences for *in vitro* cell proliferation.

Material and Methods: We studied the effects of 156 gingival crevicular fluid samples collected from subjects with different stages of periodontal disease and from healthy control subjects and the effects of MMP-1, -2, -8, -9, -13 and -14 on the degradation of EMD using EMD-embedded zymography. The effects of gingival crevicular fluid with or without EMD and the effects of amelogenin on the proliferation of cultured periodontal ligament fibroblasts were studied by cell proliferation enzyme-linked immunosorbent assay kit.

Results: Degradation of Emdogain induced by gingival crevicular fluid was greater in samples from all stages of periodontal diseases compared with healthy control samples. Of the MMPs studied, only MMP-2 and MMP-8 showed limited EMD-degrading activities. One hundred micrograms per millilitre of EMD increased proliferation of periodontal ligament fibroblasts on average by 24% (confidence interval 0.60–0.64) and at 200 μg/mL by 30% (confidence interval 0.62–0.68) compared with control fibroblasts (confidence interval 0.48–0.52). However, gingival crevicular fluid (10 μg/mL) together with 100 μg/mL EMD induced the proliferation only by 6% (confidence interval 0.51–0.55) and with 200 μg/mL EMD by 12% (confidence interval 0.54–0.58). Amelogenin at 200 μg/mL decreased the proliferation of periodontal ligament fibroblasts by 54% (confidence interval 0.22–0.25).

Conclusion: We suggest that diseased gingival crevicular fluid containing various proteases leads to degradation of EMD and decreased proliferation of periodontal ligament fibroblasts.

Timo Sorsa, Research Laboratory, Biomedicum Helsinki, Institute of Dentistry, PO Box 63, University of Helsinki FIN-00014, Helsinki, Finland
Tel: +358407374240
Fax: +358919125371
e-mail: timo.sorsa@helsinki.fi

Key words: Emdogain; amelogenin; gingival crevicular fluid; periodontitis; proliferation; matrix metalloproteinase

Accepted for publication June 15, 2009

The enamel matrix derivative Emdogain® (EMD) is a commercially available tissue extract preparation of porcine enamel origin. Over 10 years ago, it was suggested that this extract can induce new cementum and bone formation in periodontal defects in monkeys (1). Numerous studies since then have shown that EMD is clinically useful in promoting periodontal regeneration, including the restoration of alveolar bone, cementum and collagenous ligaments (2,3). Emdogain has been widely used in periodontal therapy, but the mechanisms of action and the exact composition of the product are not completely clear. It has been proposed that EMD can mimic and modify natural root development and thereby enhance regeneration (4). The predominant compound of EMD is amelogenin (> 90%; 5). In spite of amelogenin not being a growth factor, EMD possesses a number of growth factor-like effects. Emdogain has also been found to be more effective than amelogenin in enhancing fibroblast proliferation together with other cellular functions (6,7). Many studies have aspired to identify various growth factors in EMD, with varying results (8–13). Kawase *et al*. (11,12) and later Suzuki *et al*. (13) found the presence of transforming growth factor (TGF)-β1 or TGF-β-like substances in EMD. Emdogain has been documented also to contain a few other cytokines. These include a bone morphogenetic protein (BMP)-like growth factor, which belongs to the TGF-β family, and bone sialoprotein-like molecules (13,14), which have also been suggested to be the main functional components of EMD (6,11,13).

Periodontal inflammation is characterized by increased apical proliferation of gingival sulcular epithelial cells together with increased activity of proteolytic enzymes in gingival tissue and gingival crevicular fluid (15–17). Matrix metalloproteinases (MMPs) and matrix-degrading serine proteinases have been shown to be the main mediators of pathological tissue destruction in periodontitis (18–20). These proteinases, in particular MMPs, can degrade most, if not all, extracellular matrix and basement membrane components of periodontium (19–22). In periodontal treatment, a full-thickness flap procedure is used to apply the EMD in the deepest periodontal pockets and in contact with periodontally exposed root surfaces. Detectable amounts of EMD remain at the site of application on the root surface for at least 2 wks, allowing the product to be exposed to various proteolytic enzymes present in periodontal connective tissue during periodontal disease and also during surgical wound healing (23). These enzymes, produced by the cells of connective tissue and by the sulcular and attached epithelium of gingiva, are also secreted in gingival crevicular fluid (19,20).

In this study, we investigated the effects of gingival crevicular fluid, collected from patients with different stages of periodontal disease and from healthy control subjects on EMD degradation. Our hypothesis was that proteinases in gingival crevicular fluid could degrade EMD. Furthermore, we aimed to find out whether this degradation could be produced by MMPs. We also studied the effects of gingival crevicular fluid on EMD-induced proliferation of periodontal ligament fibroblasts to find out whether the degradation has any consequences *in vitro*.

Material and methods

Subjects

This study was approved by the Ethics Committee of the Ege University, Izmir, Turkey, and written informed consent, in line with Helsinki Declaration, was obtained from each subject. A total of 80 subjects (35 male, 45 female) were recruited from the Department of Periodontology, School of Dentistry, Ege University, İzmir, Turkey. Complete medical and dental histories were obtained from all subjects. Subjects were excluded if they had a history of systemic diseases and had used antibiotics and/or anti-inflammatory drugs within the last 3 mo or had received periodontal treatment within the last 6 mo. There were both smokers and non-smokers in all groups, equally distributed.

The subjects were selected according to the clinical and radiographic criteria proposed by the 1999 International World Workshop (24). The healthy control group included 13 periodontally healthy females and seven males (aged 15–61 years), with no clinical evidence of gingivitis [papillary bleeding index (25) close to 0], no radiographic evidence of bone loss and no probing depths > 3 mm. The gingivitis group included nine females and 11 males (aged 15–49 years) with bleeding on probing at any site and no radiographic evidence of bone loss (25). The chronic periodontitis group included nine females and 11 males (aged 40–63 years), who had at least four sites with a probing depth of ≥ 6 mm and attachment loss of ≥ 4 mm at the same site. Diagnosis of chronic periodontitis was made by correlating the clinical attachment loss with plaque levels. The aggressive periodontitis group included subjects with localized aggressive periodontitis (9 females and 1 male; aged 19–38 years) or generalized aggressive periodontitis (5 females and 5 males; aged 18–38 years). The localized aggressive periodontitis patients exhibited characteristic bone loss localized on the first molars and/or incisors, clinical attachment loss ≥ 4 mm in at least two permanent molars or incisors (at least one first molar affected) and up to two additional teeth, and minimal inflammation and plaque in areas other than those sites with active disease. Generalized aggressive periodontitis patients showed generalized clinical attachment loss of ≥ 4 mm on eight or more teeth; at least three of those were other than central incisors or first molars.

Gingival crevicular fluid samples

A total of 156 gingival crevicular fluid samples were collected from 80 subjects, with 20 subjects in each group. In all study groups, mesial approximal surfaces of two non-adjacent teeth were selected as gingival crevicular fluid sampling sites. In the chronic periodontitis and aggressive periodontitis groups, gingival crevicular fluid samples were collected from two approximal sites with ≥ 6 mm probing depth and ≥ 4 mm clinical attachment loss. Gingival crevicular fluid samples in the gingivitis group were collected

from two sites with bleeding upon probing and ≥ 2 mm probing depth. In the healthy group, gingival crevicular fluid samples were taken from two sites with ≤ 3 mm probing depth and without bleeding upon probing. Prior to gingival crevicular fluid sampling, supragingival plaque was removed from the interproximal surfaces using a sterile curette. These surfaces were gently air-dried and isolated with cotton rolls. Gingival crevicular fluid samples were collected by inserting filter paper strips (Periopaper; ProFlow Inc., Amityville, NY, USA) for 30 s. During this process, care was taken to avoid mechanical injury, and strips contaminated with blood were discarded. Gingival crevicular fluid volume was estimated (Periotron 8000; ProFlow Inc.), and strips were placed into a sterile polypropylene tube prior to freezing at −40°C. The study groups were blinded from the authors who did the *in vitro* assays.

Reagents

Lyophilized Emdogain and Emdogain-gel® were kindly provided by the manufacturer (Straumann, Basel, Switzerland, previously Biora AB, Malmö, Sweden), dissolved in phosphate-buffered saline stock solution (2 mg/mL) and kept at 4°C. Recombinant pig amelogenin (rP172) was expressed in *Escherichia coli* strain codon plus BL21(DE3), and isolated as described previously (26). The bacteria expressing rP172 were pelleted, resuspended in 4 M guanidine HCl (15 mL per 500 mL of culture), sonicated, and diluted with six volumes of ice-cold 0.5% formic acid. Cellular debris was pelleted by centrifugation at 4°C for 10 min (15,900 *g*). The supernatant was raised to 20% saturation with ammonium sulfate and incubated overnight at 4°C. Following centrifugation, the pellet was resuspended in ice-cold 0.5% formic acid. The supernatant was raised to 20% saturation by the addition of ammonium sulfate and incubated overnight at 4°C. The amelogenin was pelleted by centrifugation and resuspended in ammonium chloride (0.05%, pH 9–10) and passed over a phenol column (A = 0.05% AmCl; B = 80% acetonitrile + buffer A). The rP172 peak was lyophilized, resuspended in 0.05% trifluoroacetic acid (buffer C) and injected onto a preparative reverse-phase (C18) column. The amelogenin was eluted with buffer D (buffer C + 60% acetonitrile) with a gradient of 40–80% D in 185 min. The amelogenin fraction was lyophilized and stored at −20°C. The concentrations of 100 and 200 μg/mL EMD and amelogenin used in this study were selected based on our previous publication (27).

Emdogain degradation assays with gingival crevicular fluid and MMP samples

In order to detect EMD-degrading enzymes in gingival crevicular fluid, the gingival crevicular fluid samples were dissolved in TNC buffer (50 mM Tris, 0.2 M NaCl and 1 mM $CaCl_2$, pH 7.8), and the same volume (15 μL per well) of each of the 156 gingival crevicular fluid samples was loaded onto EMD-embedded zymography. Several different EMD concentrations as well as both lyophilized EMD and EMD-gel were first tested to produce as clear and high-quality gels as possible. Emdogain-gel (9 mg/mL) embedded in the 11% SDS-PAGE, instead of normally used gelatin (28), was selected to be used in these studies. We also aimed to compare the degradation profile caused by gingival crevicular fluid with the degradation profile caused by MMPs. Therefore, recombinant human MMP-1, -2, -8, -9, -13 and -14 (MMP-8: Chemicon, Termecula, CA, USA; MMP-2, -13 and -14: Invitek, Berlin, Germany; MMP-1 and -9: Calbiochem, San Diego, CA, USA) were loaded (200 ng in 3 μL per well) onto a separate EMD-embedded gel. After electrophoresis, the gels were washed twice with 2.5% Trixon X-100 buffer to renature the gelatinases. Gels were then incubated in TNC buffer for 72 h at 37°C. Gels were stained with 0.5% Coomassie Brilliant Blue R-250. The intensities of separate bands were scanned and measured quantitatively using optical densitometry and QUANTITY ONE software (Bio-Rad Model GS-700 Imaging Densitometer; Bio-Rad, Richmond, CA, USA).

Proliferation assay

Since EMD has been shown to stimulate proliferation of periodontal ligament fibroblasts (8,29–34), we aimed to study the effect of gingival crevicular fluid-induced degradation of EMD on the proliferation of periodontal ligament fibroblasts. Furthermore, the effect of recombinant amelogenin protein, which is the main compound of EMD, was studied. For the proliferation assay, human periodontal ligament fibroblasts were used. The cells were obtained from intact premolars of a healthy young male patient, by a technique described earlier (35), and cultured at 37°C in a humidified atmosphere in medium containing (per 100 mL): 90 mL Dulbecco's modified Eagle's medium, 10 mL fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomysin and 250 ng/mL Fungizone® (all supplements were from Life Technologies, Paisley, UK). The assay was carried out as previously described (36). Briefly, the cells (passage 7) were plated at a density of 5000 per well (n = 6) and allowed to attach and grow overnight. The gingival crevicular fluid samples dissolved in TNC buffer were pooled from all diseased and healthy samples and sterile filtered before adding 10 μL per well with or without EMD (100 and 200 μg/mL). Recombinant amelogenin (100 and 200 μg/mL) was used without gingival crevicular fluid. After 48 h incubation, the proliferation of periodontal ligament fibroblasts was determined with commercial Cell Proliferation ELISA, BrdU kit (Roche Diagnostics, Mannheim, Germany), where bromodeoxyuridine (BrdU) incorporation into newly synthesized DNA was measured in proliferating cells using an ELISA reader at 450 nm.

Statistical analysis

Analysis of variance models were used to estimate the effects of experimental substrates on various *in vitro* outcomes. The results are presented as relative comparisons of means between relevant treatment groups, expressed in terms of fold change or percentage from a given baseline, together with

the 95% confidence intervals (CI). Confidence intervals were calculated with the Web-based Texas A&M University's Confidence Interval Calculator (http://www.stat.tamu.edu/~jhardin/applets/index.html). Following the recommendations on statistical presentation in medical journals, we prefer to use confidence intervals in expressing statistical uncertainty instead of 'significance' testing (37).

Results

Emdogain degradation assays

The zymography with EMD-embedded gels revealed that the gingival crevicular fluid can degrade EMD (Fig. 1A). The degradation depended on the state of periodontal disease, with markedly more degradation and a wider range of molecular weights being observed in gingival crevicular fluid from periodontitis patients than in healthy gingival crevicular fluid. The analyses were carried out at the most degraded sites at 97, 50 and 37 kDa. The degradation was statistically more significant in all the disease states (Fig. 1B–D). At a molecular weight of 97 kDa, the 'EMD-lysis' in chronic periodontitis was on average 5.4-fold (mean intensity value 0.85; CI 0.65–1.05), in aggressive periodontitis 2.2-fold (mean 0.40, CI 0.22–0.49) and in gingivitis 3.9-fold (mean 0.61; CI 0.44–0.77) relative to healthy control subjects (mean 0.16; CI 0.09–0.22). The 'EMD-lysis' of 50 kDa in chronic periodontitis was on average 4.4-fold (mean intensity value 3.08; CI 2.60 – 3.56), in aggressive periodontitis 2.2-fold (mean 1.55, CI 1.11–2.00) and in gingivitis 3.3-fold (mean 2.32; CI 1.87–2.78) relative to healthy control subjects (mean 0.70; CI 0.44–0.96). The 'EMD-lysis' of 37 kDa in chronic periodontitis was on average 5.8-fold (mean intensity value 0.60; CI 0.44–0.76), in aggressive periodontitis 3.9-fold (mean 0.40; CI 0.27–0.53) and in gingivitis 3.3-fold (mean 0.35; CI 0.26–0.44) realtive to healthy control subjects (mean 0.10; CI 0.06–0.14). It is also notable that in all study groups there were samples with no observed EMD-degrading activity, but fewer in diseased groups (Fig. 1B–D).

To further determine the EMD-degrading proteases observed in periodontal gingival crevicular fluid, we studied the effects of recombinant

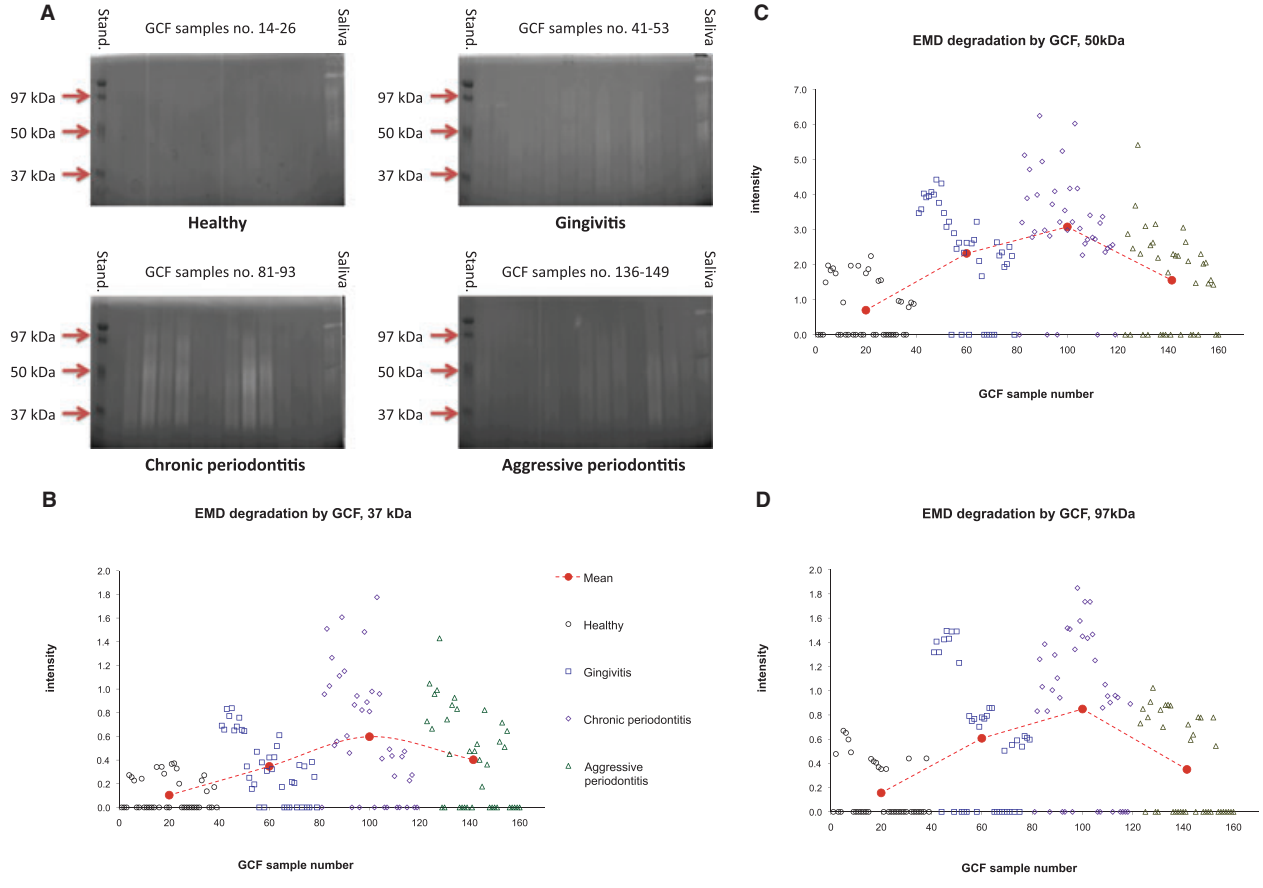

Fig. 1. Gingival crevicular fluid-induced degradation of Emdogain. In order to detect Emdogain-degrading effects of 156 gingival crevicular fluid samples obtained from subjects with different states of periodontal disease and healthy control subjects, the samples (15 μL per well) were loaded onto EMD-embedded zymography (example gels, A). The intensities of the bands were measured at the most degraded sites at 97, 50 and 37 kDa. The degradation depended on the state of periodontal disease, but the degradation was statistically more significant in all states of disease compared with the healthy control samples (B–D). The graphs (B–D) show individual scanning values of every gingival crevicular fluid sample (open symbols; $n = 39$ per group) and group means (red dots joined with a dashed line).

human MMP-1, -2, -8, -9, -13 and -14 on EMD-embedded gels. Only MMP-2 and MMP-8 produced barely detectable lysis of the gel and the bands were within a restricted range of molecular weights (Fig. 2).

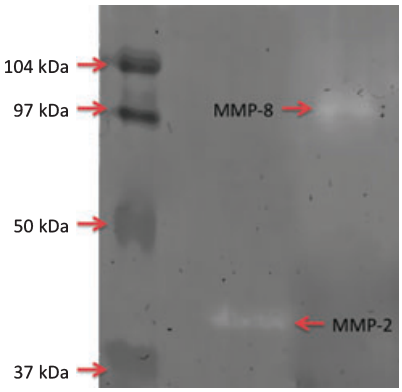

Fig. 2. Matrix metalloproteinase (MMP)-2- and MMP-8-induced degradation of Emdogain. To determine the role of MMPs on the degradation of EMD, recombinant human MMPs were loaded (200 ng in 3 μL per well) onto EMD-embedded gel. Only MMP-2 and MMP-8 caused barely detectable lysis of the gel.

Proliferation assay

The proliferation of periodontal ligament fibroblasts was remarkably induced by EMD after 48 h of incubation (Fig. 3). The induced proliferation was on average 24% higher with EMD at a concentration of 100 μg/mL, corresponding to a mean ELISA intensity of 0.62 (CI 0.60–0.64) and 30% higher (mean 0.65; CI 0.62–0.68) with EMD at a concentration of 200 μg/mL relative to control fibroblasts (mean 0.50; CI 0.48–0.52).

When EMD was incubated together with pooled and sterile filtered gingival crevicular fluid (10 μg/mL), the induction by EMD of the proliferation of periodontal ligament fibroblasts was decreased compared with treatment of cells using EMD alone (Fig. 3). Emdogain at a concentration of 200 μg/mL together with gingival crevicular fluid still increased the proliferation of fibroblasts statistically significantly by 12% (mean 0.56; CI 0.54–0.58). However, EMD at the lower concentration of 100 μg/mL together with gingival crevicular fluid induced the proliferation by 6% only, which was not significant (mean 0.53; CI 0.51–0.55) relative to the control fibroblasts. Gingival crevicular fluid (10 μg/mL) alone, without EMD, had no effect on the proliferation of periodontal ligament fibroblasts (data not shown).

No differences in proliferation were found between control and amelogenin-treated cells at a lower amelogenin concentration of 100 μg/mL (mean ELISA intensity 0.50; CI 0.47–0.53). However, amelogenin at a higher concentration had a statistically significant inhibitory effect on the proliferation of periodontal ligament fibroblasts. On average, 200 μg/mL amelogenin implied a 54% decrease (mean 0.23; CI 0.22–0.25) in the proliferation of periodontal ligament fibroblasts relative to control cells (Fig. 3).

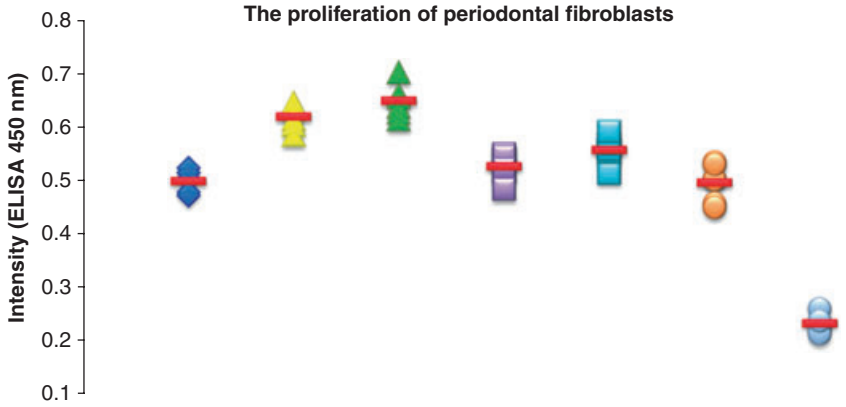

Fig. 3. The effect of Emdogain, Emdogain with gingival crevicular fluid, and amelogenin on the proliferation of periodontal ligament fibroblasts. Five thousand periodontal ligament fibroblasts per well were plated onto a 96-well microtiterplate and incubated in their normal medium overnight before adding the study reagents. The proliferation at 48 h was determined with the Cell Proliferation ELISA BrdU kit. Emdogain alone produced a statistically significant increase in proliferation of periodontal ligament fibroblasts at both studied concentrations (100 and 200 μg/mL). However, gingival crevicular fluid (10 μg/mL) together with EMD was less effective in enhancing the proliferation, and with the lower concentration of EMD (100 μg/mL) the proliferation did not differ from the untreated control fibroblasts. Amelogenin at 200 μg/mL decreased the proliferation of periodontal ligament fibroblasts drastically. The graph shows individual absorbance values of all samples and group means (blue diamonds, control; yellow triangles, EMD 100 μg/mL; green triangles, EMD 200 μg/mL; purple squares, EMD 100 μg/mL + gingival crevicular fluid 10 μg/mL; turquoise squares, EMD 200 μg/mL + gingival crevicular fluid 10 μg/mL; orange circles, amelogenin 100 μg/mL; light blue circles, amelogenin 200 μg/mL; and red lines, group mean, $n = 6$ per group).

Discussion

Our primary aim was to investigate the effects of gingival crevicular fluid on EMD. With EMD-embedded zymography, we observed that gingival crevicular fluid can, depending on the severity of the periodontal status, degrade EMD. Furthermore, we studied whether MMPs are mainly responsible for EMD degradation. However, based on our results, MMPs are not the main factors contributing to the observed degradation, and other proteases in gingival crevicular fluid are likely to be more important for the degradation of EMD *in vivo*. We also examined whether the gingival crevicular fluid-induced degradation of EMD has *in vitro* effects on the proliferation of periodontal ligament fibroblasts. The proliferation assay showed that gingival crevicular fluid depressed the proliferative effects of EMD on periodontal ligament fibroblasts. Furthermore, amelogenin did not show proliferative activity, as EMD did alone. Amelogenin at a concentration of 200 μg/mL was observed to decrease the proliferation of periodontal ligament fibroblasts, indicating that amelogenin alone

is not the proliferative component of EMD, thereby showing that other growth factor-like components of EMD might be mainly responsible for the proliferative effect of EMD on periodontal ligament fibroblasts.

In this study, we had a considerably large number of gingival crevicular fluid samples in four subgroups: healthy, gingivitis, chronic periodontitis and aggressive periodontitis. Some degree of EMD degrading activity was observed in all study groups, but significantly more in the three diseased states compared with the healthy state. Furthermore, a larger variation in degradation was observed in diseased states than in healthy samples. This may reflect the fact that healthy gingival crevicular fluid is quite homologous, containing mainly plasma components and normal tissue turnover-related proteins (38). However, the protein content of gingival crevicular fluid obtained from periodontally affected sites is different, containing more proteolytic enzymes and also proteins of non-plasma origin (38). In the present study, we loaded the same volume of gingival crevicular fluid in the gels for each gingival crevicular fluid sample, so the samples from periodontal diseased sites may contain more proteins than those from healthy subjects. However, since the *in vivo* gingival crevicular fluid volume is larger in disease, the approach of using the same volume of gingival crevicular fluid instead of the same protein amount was selected as the study method, and it may be more analogous to the *in vivo* circumstances.

The transition from a healthy state to periodontal disease is characterized by proteolytic tissue destruction. The proteolytic enzymes can be both host derived and bacteria derived (39). Initiation of periodontal degradation starts with the release of products from the bacteria. All of the periodontopathogens, in particular *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Treponema denticola*, can produce proteolytic enzymes capable of reducing the effectiveness of host defence, degrading host tissues and activating host proMMPs (40–42). However, most tissue destruction in periodontitis is mediated by host-derived proteolytic enzymes produced by sulcular epithelial cells, gingival fibroblasts, polymorphonuclear leukocytes and macrophages (18,20,21). These proteases released in gingival tissues and gingival crevicular fluid include metallo-, serine- and cysteine- proteinases (19,20,43–45). In particular, elevated levels of matrix metalloproteinase-1, -8 and -13 (collagenases) and MMP-2 and -9 (gelatinases) have been shown to correlate with periodontal disease activity (19,20). Interestingly, both metalloendoproteinase and serine protease activity have also been observed in EMD itself (46). Furthermore, EMD has been shown to have effects on the MMP levels of many cell types. Emdogain can induce a more than threefold increase in MMP-2 levels produced by periodontal ligament fibroblasts and activate the collagenase, MMP-1, in MG-63 bone-derived cells (47,48). We have previously shown that EMD can enhance MMP-2 and -9 production by HSC-3 carcinoma cells (27). Contrary to these findings, EMD has decreased the levels of MMP-1 and MMP-8 in gingival crevicular fluid after flap surgery *in vivo* (49). Since MMPs have a central role in periodontitis-induced proteolysis and EMD has been shown to be capable of regulating MMP production both *in vitro* and *in vivo*, out of the many proteolytic enzymes present in gingival crevicular fluid, we decided to study the effect of collagenases and gelatinases on the degradation of EMD. In this study, the degrading effects of MMPs on EMD-gel were limited and may be due to autolysis of the recombinant human MMPs. Thus, it seems possible that other proteolytic enzymes in gingival crevicular fluid are mainly responsible for the EMD degradation. Since the degradation caused by gingival crevicular fluid was notable over a wide range of molecular weights, it is likely that several enzymes, including certain MMPs, are acting together or even in cascade.

Emdogain consists mainly of amelogenin (> 90%), but it has been documented also to contain cytokines. Studies have found the presence of TGF-β1 or TGF-β-like substances, a BMP-like growth factor and bone sialoprotein-like molecules in EMD (11–14). Our study confirms the previous results showing that EMD can stimulate the proliferation of periodontal ligament fibroblasts *in vitro* (8,29–34). However, the recombinant amelogenin protein (200 μg/mL) was observed to depress the proliferation of periodontal ligament fibroblasts significantly. These observations suggest, as Chong *et al.* (7) have previously postulated, that amelogenin itself has very little to do with the proliferation of periodontal ligament fibroblasts, and that the stimulatory effect of EMD on the proliferation of periodontal ligament fibroblasts is caused by other factors in EMD.

In vivo, EMD has been shown to be capable of stimulating periodontal ligament regeneration in inflamed tissues (2,3). However, we observed *in vitro* that EMD together with gingival crevicular fluid was less effective in promoting the proliferation than EMD alone, and with the lower concentration of EMD (100 μg/mL) the proliferation was not different from that of the control cells. These results suggest that gingival crevicular fluid can degrade or inactivate the growth factor-like molecules in EMD responsible for proliferation. It should also be noted that due to the high consumption of gingival crevicular fluid in these assays, we had to pool the gingival crevicular fluid samples and thus the assay also contained less effective EMD-degrading gingival crevicular fluid from healthy subjects. Therefore, it may be possible that gingival crevicular fluid obtained exclusively from patients with periodontal disease can decrease the proliferation even more than pooled gingival crevicular fluid. Within the limits of this *in vitro* study, it may be suggested that to achieve the best proliferative effect of EMD on periodontal fibroblasts and therefore the best regenerative effect, it is advisable to make the infection control phase of the treatment properly before EMD application to decrease inflammation and the EMD degrading proteases present in inflamed tissues and fluids. However, further studies are needed to achieve a more precise picture of the degradation

of EMD *in vivo* and possibly to find ways for promoting the regenerative effects of EMD. To achieve this goal, *in vitro* studies of the effects of other proteolytic enzymes, in addition to MMPs, on the degradation of EMD would also be useful.

In conclusion, in this *in vitro* study we have shown for the first time that gingival crevicular fluid is capable of degrading EMD, depending on the periodontal status. Matrix metalloproteinase-2 and -8 were also found to be able to degrade EMD to a minor extent. Furthermore, gingival crevicular fluid can decrease EMD-induced proliferation of periodontal ligament fibroblasts.

Acknowledgements

The skilful technical assistance of Ms Maija-Leena Lehtonen (at the University of Oulu) is greatly appreciated. This study was supported by grants from the Academy of Finland, the Helsinki University Central Hospital Research Foundation, the Oulu University Central Hospital Research Foundation and the Finnish Dental Society Apollonia. We ensure that none of the authors have any financial or personal relationships with other people or organizations that could inappropriately influence our work.

References

1. Hammarström L. Enamel matrix, cementum development and regeneration. *J Clin Periodontol* 1997;**24:**658–668.
2. Sculean A, Schwarz F, Becker J, Brecx M. The application of an enamel matrix protein derivative (Emdogain) in regenerative periodontal therapy: a review. *Med Princ Pract* 2007;**16:**167–180.
3. Venezia E, Goldstein M, Boyan BD, Schwartz Z. The use of enamel matrix derivative in the treatment of periodontal defects: a literature review and meta-analysis. *Crit Rev Oral Biol Med* 2004; **15:**382–402.
4. Gestrelius S, Lyngstadaas SP, Hammarström L. Emdogain – periodontal regeneration based on biomimicry. *Clin Oral Investig* 2000;**4:**120–125.
5. Zeichner-David M. Is there more to enamel matrix proteins than biomineralization? *Matrix Biol* 2001;**20:**307–316.
6. Hoang AM, Klebe RJ, Steffensen B, Ryu OH, Simmer JP, Cochran DL. Amelogenin is a cell adhesion protein. *J Dent Res* 2002;**81:**497–500.
7. Chong CH, Carnes DL, Moritz AJ *et al.* Human periodontal fibroblast response to enamel matrix derivative, amelogenin, and platelet-derived growth factor-BB. *J Periodontol* 2006;**77:**1242–1252.
8. Gestrelius S, Andersson C, Lidstrom D, Hammarstrom L, Somerman M. In vitro studies on periodontal ligament cells and enamel matrix derivative. *J Clin Periodontol* 1997;**24:**685–692.
9. Newman SA, Coscia SA, Jotwani R, Iacono VJ, Cutler CW. Effects of enamel matrix derivative on *Porphyromonas gingivalis*. *J Periodontol* 2003;**74:**1191–1195.
10. Kawase T, Okuda K, Yoshie H, Burns DM. Cytostatic action of enamel matrix derivative (EMDOGAIN) on human oral squamous cell carcinoma-derived SCC25 epithelial cells. *J Periodont Res* 2000; **35:**291–300.
11. Kawase T, Okuda K, Momose M, Kato Y, Yoshie H, Burns DM. Enamel matrix derivative (EMDOGAIN) rapidly stimulates phosphorylation of the MAP kinase family and nuclear accumulation of smad2 in both oral epithelial and fibroblastic human cells. *J Periodont Res* 2001;**36:**367–376.
12. Kawase T, Okuda K, Yoshie H, Burns DM. Anti-TGF-beta antibody blocks enamel matrix derivative-induced upregulation of p21WAF1/cip1 and prevents its inhibition of human oral epithelial cell proliferation. *J Periodont Res* 2002; **37:**255–262.
13. Suzuki S, Nagano T, Yamakoshi Y *et al.* Enamel matrix derivative gel stimulates signal transduction of BMP and TGF-beta. *J Dent Res* 2005;**84:**510–514.
14. Iwata T, Morotome Y, Tanabe T, Fukae M, Ishikawa I, Oida S. Noggin blocks osteoinductive activity of porcine enamel extracts. *J Dent Res* 2002;**81:**387–391.
15. Uitto VJ, Raeste AM. Activation of latent collagenase of human leukocytes and gingival fluid by bacterial plaque. *J Dent Res* 1978;**57:**844–851.
16. Uitto VJ, Appelgren R, Robinson PJ. Collagenase and neutral metallo-proteinase activity in extracts of inflamed human gingiva. *J Periodont Res* 1981;**16:**417–424.
17. Sorsa T, Suomalainen K, Uitto VJ. The role of gingival crevicular fluid and salivary interstitial collagenases in human periodontal diseases. *Arch Oral Biol* 1990;**35:**193–196.
18. Birkedal-Hansen H. Role of cytokines and inflammatory mediators in tissue destruction. *J Periodont Res* 1993;**28:**500–510.
19. Sorsa T, Tjäderhane L, Salo T. Matrix metalloproteinases (MMPs) in oral diseases. *Oral Dis* 2004;**10:**311–318.
20. Sorsa T, Tjäderhane L, Konttinen YT *et al.* Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. *Ann Med* 2006;**38:**306–321.
21. Birkedal-Hansen H, Moore WG, Bodden MK *et al.* Matrix metalloproteinases: a review. *Crit Rev Oral Biol Med* 1993;**4:** 197–250.
22. Nagase H, Woessner JF Jr. Matrix metalloproteinases. *J Biol Chem* 1999; **274:**21491–21494.
23. Gestrelius S, Andersson C, Johansson AC *et al.* Formulation of enamel matrix derivative for surface coating. Kinetics and cell colonization. *J Clin Periodontol* 1997;**24:**678–684.
24. Armitage GC. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* 1999;**4:**1–6.
25. Saxer UP, Mühlemann HR. Motivation und Aufklärung. *SSO Schweiz Monatsschr Zahnheilkd* 1975;**85:**905–919.
26. Ryu OH, Fincham AG, Hu CC *et al.* Characterization of recombinant pig enamelysin activity and cleavage of recombinant pig and mouse amelogenins. *J Dent Res* 1999;**78:**743–750.
27. Laaksonen M, Suojanen J, Nurmenniemi S, Läärä E, Sorsa T, Salo T. The enamel matrix derivative (Emdogain) enhances human tongue carcinoma cells gelatinase production, migration and metastasis formation. *Oral Oncol* 2008;**44:**733–742.
28. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;**227:** 680–685.
29. Cattaneo V, Rota C, Silvestri M *et al.* Effect of enamel matrix derivative on human periodontal fibroblasts: proliferation, morphology and root surface colonization. An in vitro study. *J Periodontol* 2003;**38:**568–574.
30. Davenport DR, Mailhot JM, Wataha JC, Billman MA, Sharawy MM, Shrout MK. Effects of enamel matrix protein application on the viability, proliferation, and attachment of human periodontal ligament fibroblasts to diseased root surfaces in vitro. *J Clin Periodontol* 2003;**30:**125–131.
31. Okubo K, Kobayashi M, Takiguchi T *et al.* Participation of endogenous IGF-I and TGF-beta 1 with enamel matrix derivative-stimulated cell growth in human periodontal ligament cells. *J Periodontol* 2003;**38:**1–9.
32. Rincon JC, Haase HR, Bartold PM. Effect of Emdogain® on human periodontal fibroblasts in an in vitro wound-healing model. *J Periodont Res* 2003; **38:**290–295.
33. Rodrigues TL, Marchesan JT, Coletta RD *et al.* Effects of enamel matrix derivative and transforming growth factor-beta1 on human periodontal ligament fibroblasts. *J Clin Periodontol* 2007;**34:**514–522.
34. Zeldich E, Koren R, Nemcovsky C, Weinreb M. Enamel matrix derivative

stimulates human gingival fibroblast proliferation via ERK. *J Dent Res* 2007; **86:**41–46.
35. Oikarinen K, Seppa S. Effect of preservation media on proliferation and collagen biosynthesis of periodontal ligament fibroblasts. *Endod Dent Traumatol* 1987; **3:**95–99.
36. Heikkilä P, Suojanen J, Pirilä E *et al*. Human tongue carcinoma growth is inhibited by selective antigelatinolytic peptides. *Int J Cancer* 2006;**118:**2202–2209.
37. Sterne J, Davey Smith G. Shifting the evidence – what's wrong with significance tests. *BMJ* 2001;**322:**226–231.
38. Curtis MA, Sterne JA, Price SJ *et al*. The protein composition of gingival crevicular fluid sampled from male adolescents with no destructive periodontitis: baseline data of a longitudinal study. *J Periodont Res* 1990;**25:**6–16.
39. Sandholm L. Proteases and their inhibitors in chronic inflammatory periodontal disease. *J Clin Periodontol* 1986; **13:**19–26.
40. Potempa J, Banbula A, Travis J. Role of bacterial proteinases in matrix destruction and modulation of host responses. *Periodontol 2000* 2000;**24:**153–192.
41. Eley BM, Cox SW. Proteolytic and hydrolytic enzymes from putative periodontal pathogens: characterization, molecular genetics, effects on host defenses and tissues and detection in gingival crevice fluid. *Periodontol 2000* 2003;**31:**105–124.
42. Ding Y, Uitto VJ, Firth J *et al*. Modulation of host matrix metalloproteinases by bacterial virulence factors relevant in human periodontal diseases. *Oral Dis* 1995;**1:**279–286.
43. Eley BM, Cox SW. Cathepsin B/L-, elastase-, tryptase-, trypsin- and dipeptidyl peptidase IV-like activities in gingival crevicular fluid: a comparison of levels before and after periodontal surgery in chronic periodontitis patients. *J Periodontol* 1992;**63:**412–417.
44. Palcanis KG, Larjava IK, Wells BR *et al*. Elastase as an indicator of periodontal disease progression. *J Periodontol* 1992; **63:**237–242.
45. Lah TT, Babnik J, Schiffmann E, Turk V, Skaleric U. Cysteine proteinases and inhibitors in inflammation: their role in periodontal disease. *J Periodontol* 1993; **64:**485–491.
46. Maycock J, Wood SR, Brookes SJ, Shore RC, Robinson C, Kirkham J. Characterization of a porcine amelogenin preparation, EMDOGAIN, a biological treatment for periodontal disease. *Connect Tissue Res* 2002;**43:**472–476.
47. Mirastschijski U, Konrad D, Lundberg E, Lyngstadaas SP, Jorgensen LN, Agren MS. Effects of a topical enamel matrix derivative on skin wound healing. *Wound Repair Regen* 2004;**12:**100–108.
48. Goda S, Inoue H, Kaneshita Y *et al*. Emdogain stimulates matrix degradation by osteoblasts. *J Dent Res* 2008;**87:**782–787.
49. Okuda K, Miyazaki A, Momose M *et al*. Levels of tissue inhibitor of metalloproteinases-1 and matrix metalloproteinases-1 and -8 in gingival crevicular fluid following treatment with enamel matrix derivative (EMDOGAIN). *J Periodont Res* 2001;**36:**309–316.