Detection of Prostaglandin E_2 and Matrix Metalloproteinases in Implant Crevicular Fluid

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Studies show that implants exhibiting peri-implantitis contain elevated levels of the cytokine interleukin-1ß in the gingival crevicular fluid (GCF). This study further evaluated possible mechanisms of osseous loss in peri-implantitis by examining GCF samples for the presence of prostaglandin E_2 (PGE_2) and proteolytic enzymes, specifically matrix metalloproteinases (MMPs). Results indicated that levels of PGE_2 in healthy sites were not significantly different from those at diseased sites. MMP species migrated at 92 kd and 66 kd. No qualitative difference in bands was seen between healthy implants and those diagnosed with early peri-implantitis. Results suggested that PGE_2 and MMP levels are not useful biologic markers for distinguishing between healthy and diseased implants. (INT J ORAL MAXILLOFAC IMPLANTS 1998;13:689–696)

Key words: cytokines, gingival crevicular fluid, matrix metalloproteinases, peri-implantitis, prostaglandin E_2

The predictability of osseointegrated implants for the successful replacement of missing teeth has resulted in their widespread use.[1–3] However, it has become increasingly evident that complications involving pathologic changes in the peri-implant tissue can occur following osseointegration and loading in a small percentage of cases. Failing implants develop inflammatory changes in the soft tissue surrounding the implant; these changes are accompanied by a progressive peri-implant bone loss that is similar to periodontitis.[4] This process is known as peri-implantitis.

Peri-implantitis is the result of the host response to plaque accumulation at the implant site. As in any inflammatory state, activated leukocytes produce a variety of proteins that alter cellular activity. These proteins, including inflammatory cytokines and proteolytic enzymes, mediate the host response to exogenous antigens.[5] Previous studies have shown that inflammatory cytokines such as Interleukin-1 beta (IL-1ß), which are released by host monocytes and macrophages in response to bacterial products such as lipopolysaccharide (LPS) and endotoxin, play a role in the breakdown of the periodontium in periodontitis.[6,7] IL-1ß has been found to induce prostaglandin E_2 (PGE_2) synthesis by macrophages and gingival fibroblasts. PGE_2, a vasodilator, increases vascular permeability at sites of inflammation and mediates bone resorption. It has been assayed in gingival crevicular fluid (GCF) from teeth with active periodontal disease.[8] IL-1ß has also been shown to stimulate proteolytic enzyme production in macrophages, osteoblasts,[9,10] fibroblasts,[11] and chondrocytes.[12] These proteolytic enzymes, known as matrix metalloproteinases (MMPs), contribute to the degradation and removal of collagen from damaged tissue; they are secreted by cells that reside in the inflammatory site in response to stimuli such as LPS or cytokines.[13]

While the inflammatory reaction is a defensive response to components of microbial plaque including endotoxins and LPS, it is now clear that these same inflammatory mediators that provide protection from bacterial infection may also be responsible for most of the tissue damage.[14] The role of these

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inflammatory mediators in the pathogenesis of periodontal disease around natural teeth has been previously studied[6,7,13–19]; however, the question of whether similar pathogenesis is responsible for the tissue destruction that occurs around implants is still not clearly established.

A recent study demonstrated that GCF from implants diagnosed with peri-implantitis contains elevated levels of the inflammatory cytokine IL-1ß.[20] These results were similar to those reported for IL-1ß levels in periodontitis, supporting the belief that the two disease processes resemble each other both in etiology and pathogenesis. Rams et al[21] has shown that crevicular leukocytes are found in increased numbers in patients whose implants are considered to be failing. Adell et al[22] found plasma cells, lymphocytes, polymorphonuclear leukocytes, and macrophage infiltrates in peri-implant soft tissue. Prostaglandins, especially PGE_2, are potent local regulators of bone metabolism.[23] IL-1ß has been shown to induce the production of prostaglandins by macrophages and fibroblasts in the periodontal tissues.[11] Tissue collagenase activity in both the gingiva and the mucosa of implants and teeth with increased presence of inflammation has also been observed.[24] Thus, these findings suggest that a similar mechanism involving inflammatory mediators may also be responsible for the pathogenesis of peri-implantitis.

The focus of this study was to continue the investigation into the mechanism of osseous loss in peri-implantitis by examining GCF samples for the presence of proteolytic enzymes, specifically MMPs, and PGE_2. Detection of the presence and levels of these mediators in the implant inflammatory response may provide insight into their possible role in peri-implantitis.

Materials and Methods

Patient Selection. Patients were recruited from the graduate prosthodontic clinic at the New Jersey Dental School. Subjects were excluded from the study if they were not medically healthy and/or were taking medication that could influence peri-implantitis. These exclusions involved patients requiring endocarditis prophylaxis and patients who had used 0.12% chlorhexidine rinse, systemic antibiotics, cortisone, or nonsteroidal anti-inflammatory drugs within the previous 3 months. For inclusion in the study, patients' endosseous implants must have been loaded for a minimum of 6 months. Informed consent was obtained in writing from each subject prior to initiation of the study.

At the initial visit, a comprehensive medical and dental history was obtained. All patients received a complete oral exam that included notation of mucosal abnormalities, assessment of the plaque index and gingival index, and measurement of pocket depth both at existing dentition and at the implant site(s) when inflammation was present. A radiograph of the implant was taken if existing radiographs were more than 90 days old. Implants deemed as failures and planned for removal were not included in the study. Each implant was assigned to a diagnostic category (healthy or early peri-implantitis) based on the following clinical and radiographic criteria:

1. *Healthy:* no gingival inflammation as evidenced by lack of localized erythema or bleeding on probing; no pockets greater than 4 mm; attachment loss no greater than 2 mm; and no evidence of radiographic bone loss
2. *Early Peri-implantitis:* presence of gingival inflammation; bleeding on probing; no pockets greater than 6 mm; loss of attachment no greater than 2 mm; evidence of bone loss not exceeding 30%; and no mobility.

GCF Sample Collection. From a population of 29 implant patients treated at the New Jersey Dental School, 37 healthy implants and 37 implants affected with early "implantitis" were selected for the study. Samples of GCF were randomly collected from one of four possible sites around each implant: the mesial, distal, facial/buccal, or lingual/palatal surfaces. Samples were collected before any probing measurements were taken to prevent contamination of the strips with blood. To collect samples, a filter paper strip (Proflow, Amityville, NY) was inserted into the gingival crevice for 30 seconds. The strip was placed into a labeled Eppendorf microcentrifuge tube in ice and stored at –20°C until assayed.

Sample Preparation. Samples were eluted from the paper strips by the addition of 215 µL of sterile phosphate-buffered saline containing 0.1% bovine serum albumin to each tube, which then incubated overnight at 4°C.

Cytokine Assay. IL-1ß levels were determined by means of an enzyme-linked immunosorbent assay (ELISA) kit (Cistron, Pine Brook, NJ) using 37 samples. A total of 100 µL of eluted sample was assayed. A microplate reader was used to read the results, and the concentration of the cytokine was determined by generating a standard curve for comparison. Concentrations of cytokines were corrected for eluate volume and reported as pg/site.

Prostaglandin Assay. An assay was also conducted for the prostaglandin PGE_2 by means of an enzyme immunoassay kit (EIA) (Advanced Magnetics, Cambridge, MA) using the 37 samples used to

Table 1 Mean Levels of IL-1ß and PGE_2 at Healthy and Diseased Implant Sites

Health status	No. of sites	IL-1ß (pg/site ± SEM)	PGE_2 (pg/site ± SEM)
Healthy	37	13.0 ± 5.2	986.0 ± 68.8
Early peri-implantitis	37	609.5 ± 54.3	1085.9 ± 79.3

Table 2 Statistical Analysis*

Variables (pg/site)	*t*-value	*df*	Two-tail significance	95% CI
IL-1ß	–10.94	72	.000	–705.09, –487.71
IL-1ß (log)	–19.32	72	.000	–6.117, –4.793
PGE_2	–.94	72	.349	–308.42, 110.43
PGE_2 (log)	–.71	72	.483	–.116, .055

**t* test; $P < .001$; 95% confidence interval for difference.

determine levels of IL-1ß. A total of 50 µL was assayed from these samples, and the concentration of prostaglandin was determined by standard curve comparison. Concentrations were corrected for eluate volume and reported as pg/site.

Proteolytic Enzyme Assay (MMP). Levels of MMP in the eluted samples were determined by means of substrate polyacrylamide zymography,[25] which consists of routine sodium-dodecyl substrate polyacrylamide gel electrophoresis with a substrate for the enzyme(s) of interest copolymerized into the gel. MMP activity was determined by using 2.0% gelatin as the copolymerized substrate. Thirty-two samples taken from 16 healthy and 16 early peri-implantitis sites were analyzed. The MMPs that have gelatinase activity and can be detected in these zymograms are MMP-2/72 kd gelatinase, MMP-3/stromelysin, MMP-7/uterine MMP, MMP-9/92 kd gelatinase, and MMP-10/stromelysin-2. After 10 µL of each sample was electrophoresed, the gels were incubated at 37°C for 24 hours in 50 mmol/L tris-hydrochloric acid, pH 7.5, 5.0 mmol/L calcium chloride, 1.0% Triton X-100, and 0.02% sodium azide. The gels were stained with 0.2% amido black; destained MMP activity was then visualized as clearance zones in the stained gels.

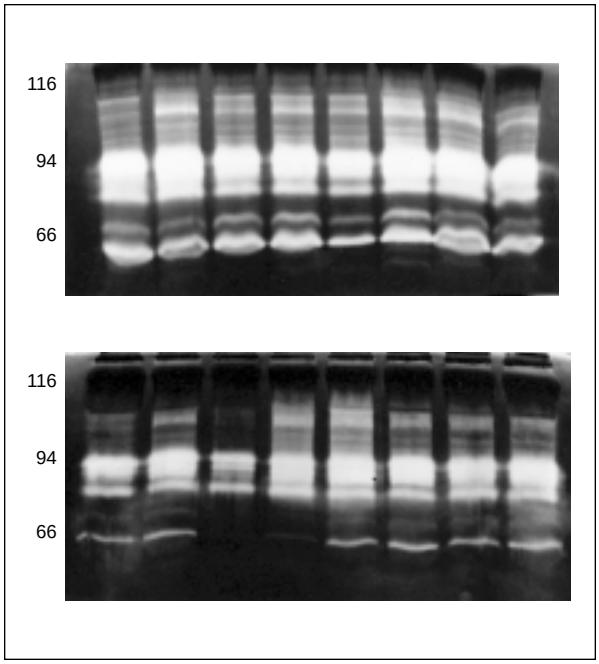
Results

The aim of this study was the examination of implants diagnosed with peri-implantitis for the purpose of determining the presence and role of immunomodulators such as PGE_2 and MMP in its pathogenesis. The results indicate that the levels of PGE_2 in healthy sites were not significantly different from those in diseased sites. Comparing the mean PGE_2 levels with the mean IL-1ß levels in these implants showed that the IL-1ß increased by sixfold in early peri-implantitis versus healthy implants. Meanwhile, no significant difference was found in the PGE_2 levels around healthy and peri-implantitis implants (Table 1). Based on these results, no correlation could be made between PGE_2 and IL-1ß levels in the same samples.

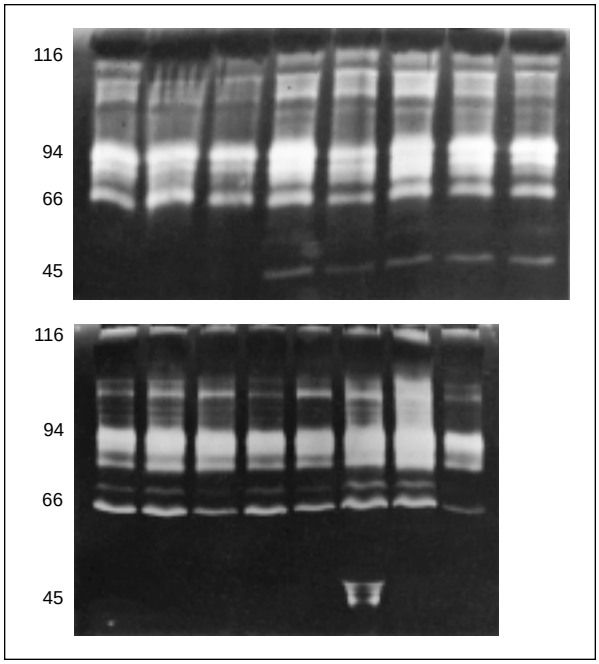
Analysis of the IL-1ß and PGE_2 data was first done using an unpaired Student's *t* test. However, closer evaluation of the data revealed that they were not normally distributed; therefore, a logarithmic transformation of the data was carried out to normalize the data points, and an unpaired Student's *t* test was again used. Both analyses confirmed the results of the comparison of the means, that is, a significant difference ($P < .001$) existed between the healthy and peri-implantitis groups of IL-1ß, but not between the two groups of PGE_2 (Table 2).

The two implant types (Brånemark, Nobel Biocare, Westmont, IL, and Calcitek, Carlsbad, CA) were equally distributed in the healthy and diseased groups. No difference was seen in the levels of IL-1ß for either implant type. However, this lack of difference may be the result of the small sample size analyzed. A larger sample population may have revealed a difference. Further work will need to be done to address a possible difference in implant types.

Analysis of the GCF samples using gel zymography to determine the proteolytic enzyme profile of each implant found that MMPs were not reliable indicators of implant health. The gel obtained from implants that were diagnosed as healthy according to their periodontal parameters and that registered low levels of IL-1ß had an MMP species with a strong band appearing around 92 kd, which corresponds to MMP-9 (Figs 1a and 1b). A band was also discernible at 66 kd, which corresponds to MMP-2. Upon exami-



Figs 1a and 1b Substrate acrylamide gel zymography of 10-µL eluate of 16 GCF samples from healthy implants. A major species of protein with a strong band was found to migrate to 92 kd, corresponding to MMP-9. A band also discernible at 66 kd corresponds to MMP-2.



Figs 2a and 2b Substrate acrylamide gel zymography of 10-µL eluate of 16 GCF samples of implants with early peri-implantitis. A major species of protein was found to migrate to 92 kd (MMP-9) and 66 kd (MMP-2).

nation of the implants with early peri-implantitis, it was again found that the major species migrated to 92 and 66 kd (Figs 2a and 2b). Some minor banding was also detected at both 110 and 45 kd in peri-implantitis samples, which may be of some importance and warrants further investigation. However, proteolytic enzyme levels around the implants showed no significant qualitative difference between the healthy and diseased sites.

Discussion

It has been established that, like cytokines, PGE_2 and MMP are found in crevicular fluid and are believed to play an active role in periodontal destruction.[6,19,26] In addition to IL-1ß and tumor necrosis factor (TNF-α), there is evidence demonstrating the importance of the products of arachidonic acid metabolism, especially the prostaglandins, in tissue destruction associated with periodontal disease.[27–29] Prostaglandins, especially PGE_2, are potent local regulators of bone metabolism.[23] PGE_2 has been found in inflamed periodontal tissue and in GCF, and at higher levels in active disease sites.[30] IL-1ß has been shown to induce the production of prostaglandins by macrophages and fibroblasts in periodontal tissues.[11] Sato et al[31] studied the effects of IL-1ß on an osteoblastic cell line and showed that IL-1ß can stimulate "macrophage-colony stimulating activity" and PGE_2 production. These and other studies[29] suggest that the effect of IL-1ß on bone resorption is mediated in part by PGE_2 production. Prostaglandin production not only plays a role in bone resorption, but also has the capacity to dampen or suppress the production of IL-1ß and TNF-α, thereby regulating the character of the inflammatory response by a feedback loop.[27–30]

Based on these investigations, the role of PGE_2 in the pathogenesis of peri-implantitis was studied by measuring its levels in collected GCF. The results indicate that the levels of PGE_2 in healthy versus diseased sites were not significantly different (Fig 3). Thus, no correlation could be made to the levels of IL-1ß present in the same samples. One possible explanation for these results could be that PGE_2 was not stimulated in the diseased sites and therefore did not contribute to the inflammatory process. Another explanation is that TNF-α is a more potent stimulator of PGE_2 than IL-1ß, and since TNF-α was not detected in the samples from a previous study,[20] it follows that PGE_2 also did not show a significant difference between healthy and diseased sites.

It is important to note that PGE_2 has also been shown to stimulate bone formation.[32] The role of PGE_2 in the regulation of bone resorption and bone

formation[31,32] may explain the slightly higher level found in the diseased implants. However, the clinically indiscriminative levels found around healthy and diseased implants may mean that PGE_2 maintains the homeostasis of the local environment (healthy or diseased) and cannot be used as a marker for disease activity. This finding and reasoning needs to be confirmed in future studies, possibly by analysis of a larger population sample.

It has also been found that MMPs, like IL-1ß and PGE_2, are found in crevicular fluid and also play an active role in periodontal destruction.[33] MMPs comprise a family of enzymes that can cleave the triple helix of collagen and collectively degrade the extracellular matrix in chronic inflammatory diseases such as periodontitis. These metalloproteinases are secreted in a precursor form that requires activation and are inhibited by tissue inhibitors of metalloproteinases (TIMPs).[34,35] The MMPs that have been studied appear to be metalloenzymes that are active in the presence of divalent metal cations, such as Zn^{2+} and Ca^{2+},[36] and that vary in molecular size, ranging from 25 kd up to 100 kd.[37] MMPs can be broadly classified as: *(1)* collagenases (type I; fibroblast-type and PMN-type collagenase); *(2)* gelatinases (type IV collagenases; 72 kd and 92 kd gelatinase); *(3)* stromelysins (stromelysins-1, -2, -3); and *(4)* other proteinases (macrophage metalloelastase and matrilysin).[38] Type I collagenase (the major predominant form of collagenase in diseased periodontal tissue[34,35,39]) cleaves collagen into two fragments: a larger 75% fragment, and a shorter 25% fragment[38] comprising the active form of collagenase in the diseased periodontal tissue.[34,35,39] Once the collagen molecule is cleaved, the triple helix unravels, becoming soluble and exposing itself to further degradation by other proteases.

The MMPs and other enzymes that degrade connective tissue are secreted by a variety of cell types, including macrophages, polymorphonuclear leukocytes, fibroblasts, and osteoblasts.[34,40] The enzymes that degrade the periodontal connective tissues are produced primarily by resident fibroblasts and macrophages.[14] In periodontal disease, loss of attachment is associated with the breakdown by MMPs of collagen fibers in the periodontal tissues. As the primary lesion progresses, collagen degradation occurs in the gingiva, periodontal ligament, and alveolar bone. Active and latent MMPs (also known as pro-MMPs) have been detected in chronically inflamed gingival tissue and in GCF samples.[9,10,29,40]

Concentrations as low as 1 ng/mL of IL-1ß have been found in in vitro studies to induce the synthesis and secretion of metalloproteinases, including collagenase, gelatinase, and stromelysin, with a concomitant reduction in the production of TIMP.[29,34] Meikle et al[29] demonstrated the role of TNF-α and IL-1ß in tissue destruction and produced evidence that breakdown is mediated by MMPs. Collagenolysis was found to depend on the dose of TNF-α and IL-1ß, with collagen degradation occurring and accompanied by increased synthesis and release of the MMPs, collagenase, gelatinase, and stromelysin. The results demonstrated the important roles of MMP in connective tissue destruction and support the hypothesis that such destruction during an inflammatory disease may be partly initiated by the cytokines in the TNF-α and IL-1ß.

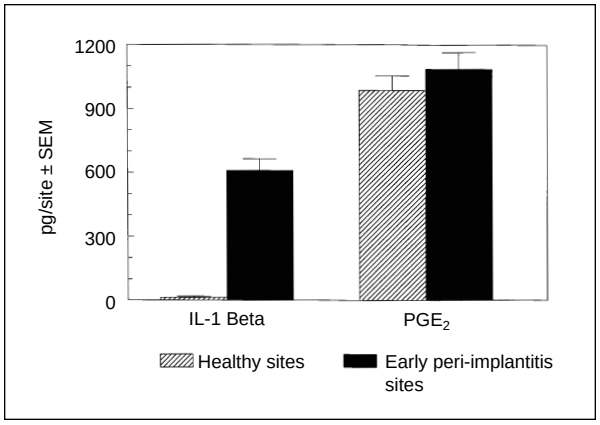


Fig 3 IL-1ß and PGE_2 levels in GCF samples of healthy implant sites and at early peri-implantitis sites.

Grant et al[35] found a variety of in vitro agents and stimuli that are capable of activating these MMPs by disrupting the structure of the stable latent enzyme that allows the active proteolytic site to become exposed. Once activated, these cells are capable of producing not only proteins with proteolytic function, with the ability to degrade all of the components of the connective tissue matrix, but also other enzymes capable of causing the MMPs to be activated from the latent form.

Meikle et al[29] have proposed that a major cause of tissue destruction in periodontal disease is the interaction between bacterial antigens and the inflammatory cells. This leads to the production of IL-1ß and TNF-α, which in turn stimulates MMP production and thus degradation of the periodontium.[29] Birkedal-Hansen et al[41] found that periodontal microbial pathogens on cultured epithelial cells can directly produce factors that stimulate degradation of collagen fibrils. Hence, it is possible to activate the metalloproteinases either directly via the presence of pathogenic bacteria or indirectly via stimulation of host cells to produce cytokines, which in turn induce local cells to produce collagenase. Active and latent

collagenases have been detected in GCF samples.[9] Villela et al[40] were able to demonstrate that little or no collagenase is released at clinically healthy sites of the periodontium, but increased with disease severity at diseased sites. Other studies[10,39] also found a positive correlation between the level of active collagenase in crevicular fluid and periodontal destruction.

Sodek and Overall[42] have presented strong evidence for the involvement of MMP in the resorption of bone and in the inflammation-mediated destruction of the periodontal tissues. They explained how osteoblasts, under the influence of osteotropic harmones (vitamin D_3, parathyroid hormone), produce MMPs that appear to remove the unmineralized osteoid layer, which provides a physical barrier to the osteoclast, preventing these cells from attaching to the mineralized tissue. Sodek and Overall's study was supported by Vaes et al,[33] who had a similar explanation for the role of MMP in bone resorption. Thus the analysis of these proteinases and inhibitors in crevicular fluid may provide a potentially useful diagnostic marker for the assessment of tissue health and implant success. This may allow for intervention and treatment before clinical failure of the implant.

The role of MMP and protease activity in the peri-implant sulcus has not yet been clearly defined. Eley et al[43] examined the peri-implant sulcus fluid from patients with permucosal osseointegrated implants and measured the protease activities in the eluates. They found that peri-implant sulcular fluid proteases may be valuable in monitoring tissue responses to implants. Apse et al[44] also looked at collagenase activity in the GCF secreted around implants and found that all of the osseointegrated implant sites contained both active collagenase and procollagenase, despite the appearance of relatively healthy soft tissues. The presence of MMP-5 (identical to MMP-2, type IV collagenase; 66-72 kd) was also found, as was evidenced by cleavage of native 75% collagen fragments.[44]

These studies, along with the increased levels of IL-ß, found in association with peri-implantitis in the present investigation, prompted the evaluation of the presence of MMP and determination of their possible role or contribution to inflammation/destruction. Examination of MMP in GCF samples from both healthy and diseased (early peri-implantitis) sites found no significant qualitative difference between the two groups. The major band on all examined samples migrated at approximately 92 kd, corresponding to the migration pattern of MMP-9. A slightly more intense band was observed at 92 kd, in the healthy samples compared to samples with peri-implantitis. In implants exhibiting peri-implantitis, the presence of MMP-2 (around 68 kd) was slightly more prominent. In addition, a few samples from diseased implants displayed bands migrating at 45 kd. However, findings indicated that there was no overall significant qualitative difference noted in band intensities for implants at healthy sites and those exhibiting peri-implantitis.

The type IV collagenases/gelatinases (MMP-2 and MMP-9) are part of a specific subgroup of the metalloproteinase family that degrade type IV collagen and denatured type I collagen (gelatin).[37] Gelatinase (molecular weight ranging from 60 to 90 kd) is secreted as a latent enzyme, activated by proteolysis, and inhibited by TIMP.[37] Perhaps the reason for the slightly more intense bands in healthy patients is related to activation of the samples during analysis. It is also important to note that the elution buffer used in this investigation was phosphate-buffered saline and bovine serum albumin, which are of 64 kd molecular weight and could possibly mask the bands that appear around 60 to 66 kd. The 45 kd bands possibly were a degradation product of the 68 kd band, or they could represent MMP-3/stromelysin.

Confirming the work of Eley et al,[43] this study identified the presence of MMP in the peri-implant GCF. However, as with Apse's study,[44] the proteolytic enzyme profile could not be used qualitatively as an indicator of implant health since it did not vary between healthy and diseased implants. This finding raises the question of whether destructive enzymes, which potentially can be induced by proinflammatory cytokines, are possibly newly released inflammatory cell (polymorphonuclear-leukocyte, macrophages) products in the crevicular fluid or intact inside the cells at the time of sampling and released only during sample preparation. Another explanation may be that one would not expect to see very high levels of MMP, since tissue destruction would be extensive if the levels were very high, resulting in destruction of a tooth in the natural dentition. When present, high levels may appear only at the beginning of the inflammatory process and then decrease with time. Therefore, if measurement is made after the level has decreased, it may be difficult to detect a difference between healthy and diseased sites. The answer may also be a combination of both situations, which would account for the comparable findings at healthy and diseased peri-implant sulci. The fact that implant sites with increased levels of IL-1ß did not have a distinctly more prominent appearance of MMPs, as has been observed in in vitro studies,[12,33–38,41–45] may also be related to the regulation and control of metalloproteinase activity and inactivity based on the availability, half-life, and relative concentrations of the cytokine.

Although the in vitro activation of MMP has been described in detail, the "pathways" of in vivo activation are still not understood and warrant further

investigation. The availability and concentration of TIMP also need to be considered when determining the extent and duration of the role of MMP in degradative activity. TIMP is produced concurrently with the latent forms of the metalloproteinases[26] and has the capacity to inhibit the metalloproteinases that degrade extracellular matrix components. In the periodontium of natural teeth, TIMP was found in higher concentrations at healthy sites than at highly inflamed periodontal sites, which demonstrated increased enzyme levels.[10] The fact that TIMP values, with their possible influence on the regulation of enzyme activity, were not examined in this study leaves the findings regarding MMP open to further investigation. This was also a qualitative study to determine the presence of MMP around healthy or diseased implants; a more critical analysis of MMP levels should be done using a more sensitive assay, such as an antibody Western Blot for a quantitative analysis. Thus, additional experimentation is needed to clarify the role of MMP in the destruction of the peri-implant tissue before it can be used as a marker of tissue health.

Conclusion

Peri-implantitis is usually diagnosed based on patients' complaints and managed by means of subjective clinical findings that indicate the extent of pathology.[46] However, in asymptomatic patients, it becomes difficult to determine if present clinical and radiographic findings represent active disease or evidence of past disease activity. This determination is critical for proper management of the peri-implant tissue. In a previous study, the use of IL-1ß as a viable marker for detection of disease has been found to be plausible, since increased crevicular levels of IL-1ß were found in implants with an initial or latent disease process when clinical parameters revealed no such activity. However, in this investigation of PGE_2 and proteolytic enzymes involved in peri-implant destruction, specifically MMPs, no correlation between health and disease in implants was found. Thus, measurement of IL-1ß in implant GCF may offer a more reliable method for routine detection of active peri-implant disease, as well as evidence that disease management of the infection is required.[47] Further work into the quantitative values of MMP found in healthy or diseased implants is necessary.

Acknowledgments

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