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Matrix metalloprotinase-8 activity in gingival crevicular fluid: Development of a novel assay

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Abstract

Objective: The matrix metalloproteinases (MMPs) play a role in regulating turnover and metabolism of connective tissues in health but they have also been implicated in a wide variety of pathological conditions including periodontal disease. MMP-8 has been extensively studied in periodontal health and disease using ELISA, although this technique is limited by its inability to determine enzyme activity. The aim was to develop and assay to specifically measure MMP-8 activity and to demonstrate its use in the analysis of gingival crevicular fluid samples.

Materials and Methods: A specific antibody was used to coat black 96 well microtitre plates to capture MMP-8 selectively. The activity of bound MMP-8 was measured using a fluorogenic substrate. GCF samples, from healthy and periodontally diseased sites, were collected using Perio-paper strips and tested for MMP-8 activity.

Results: Significantly higher MMP-8 activity was demonstrated in GCF from periodontally diseased sites compared with healthy sites, which exhibited basal or no MMP-8 activity. No cross-reactivity with other MMPs was noted.

Conclusion: We show for the first time that MMP-8 activity can be specifically detected and quantified in GCF samples. Measurement of MMP-8 activity could prove to be useful in monitoring periodontal disease progression.

Introduction

Periodontal disease is a chronic inflammatory disease resulting in the destruction of the periodontal ligament and alveolar bone and can ultimately lead to tooth loss (1). Although bacterial plaque initiates the disease process, the majority of the tissue destruction in periodontitis is associated with host rather than bacterial proteinases (2). Host proteinases, such as the matrix metalloproteinases (MMPs), a family of over twenty structurally related zinc endopeptidases, are collectively known to have the ability to degrade all components of the extracellular matrix and basement membrane. As such the MMPs have well described proteolytic roles in tissue destruction in a variety of disease states, including oral diseases (reviewed by 3, 4).

The MMPs are numbered in terms of their order of discovery and are classified on the basis of their preferred substrates. The five major substrate specific MMP groupings are: the collagenases (MMP-1, MMP-8, MMP-13), the gelatinases (MMP-2, MMP-9), the stromelysins (MMP-3, MMP-10, MMP-11), the matrilysins (MMP -7, MMP-26) and the membrane type MMPs (transmembrane type; MMP-14, MMP-15, MMP-16, MMP-24: or GPI-anchor type; MMP-17; MMP-25) (5). Nonextracellular matrix substrates for MMPs are also emerging (6) and it is accepted that the MMPs have multifunctional roles as diverse as cytokine processing (7) and modulation of growth factor bioavailability (8). Basal production of MMPs is generally low, as transcription is closely and individually regulated by a variety of growth factors and cytokines (9, 10). Furthermore, recent evidence also suggests MMP expression is regulated post-translationally *via* microRNAs (11). In addition, the enzymatic activity of MMPs is influenced by their specific localisation (or compartmentalisation) (12) and by the process of MMP pro-enzyme or zymogen activation (13). The relative abundance of endogenous inhibitors, such as the TIMPs (14, 15) and the broad spectrum proteinase inhibitor α_2-macroglobulin (16) provide the final level of control of local MMP activity. Given the complexity of MMP regulation, it is important to appreciate that estimation of mRNA or protein levels of the enzymes may not correspond with MMP activity. Furthermore, because MMPs have overlapping substrate specificities, it is difficult to design substrates that are specific for a single MMP. Thus, novel assays, that combine the specificity of antibody based approaches with the ability to measure enzyme activity using a fluorescent substrate, are required if the activity levels of individual MMPs are to be quantified successfully.

In periodontitis, neutrophils attracted to sites harbouring bacterial biofilms are a major source of MMPs, particularly MMP-8 (released from specific/secondary granules) and MMP-9 (from secretory/tertiary granules) (17). MMP-8 released from neutrophils has been characterised to unwind

the gross triple helix conformation of collagen and thus initiate collagen destruction, whereas MMP-9 can only degrade collagen which has already been denatured by the collagenases (18). The MMPs have emerged as a family of proteolytic enzymes worthy of investigation as both diagnostic aids (19-23) and as therapeutic targets in periodontitis (24). Although active MMPs contribute to the ongoing tissue destruction in periodontal disease, antibody-based commercial ELISAs are commonly used to quantify MMP levels, rather than the more clinically relevant quantification of MMP activity (25). Although more sensitive immunofluorometric assays (IFMAs) have also been employed (23, 26, 27), these also rely exclusively on antibody-based detection. Since antibody-based methods detect all forms of MMP, including TIMP-inhibited MMP, inactive proMMP and active MMP, more advanced MMP assays are required for quantification of enzymatically active forms of MMPs.

MMP-8 has an important role in the initial stages of collagen degradation. It has also been shown to be highly discriminatory for the site-specific diagnosis of periodontitis (21, 23, 26) and the longitudinal monitoring of periodontitis (28). Since measuring MMP-8 abundance by ELISA does not correspond to enzyme function, the aim of this study was to develop an improved, more clinically relevant assay to specifically quantify active MMP-8 levels in GCF samples.

Materials and methods

Subjects

Ethical approval for the study was obtained from the Office for Research Ethics Committees Northern Ireland (ORECNI) and written consent was obtained from all participants for the collection of GCF samples. Patients attending the Periodontal clinic, School of Dentistry, Belfast were recruited to the study. All patients were diagnosed with chronic periodontitis according to the 1999 International Workshop on the Classification of Periodontal diseases and conditions (29). Patients were aged between 40 and 65 years old, had a minimum of 16 teeth and at least 8 teeth with sites exhibiting probing depths ≥5mm.

Collection of gingival crevicular fluid

GCF was collected as described previously (30) from a total of 15 periodontal healthy sites (from five periodontally healthy subjects) and 10 periodontally diseased sites (from nine individual patients). Briefly, teeth to be sampled were dried with warm air for 10 s. and supragingival deposits of plaque were removed. A strip of perio-paper (Periopaper®; Proflow, Amityville, NY, USA) was inserted into the gingival crevice until light resistance was felt and was then left in place for 30 s. Perio-papers contaminated with blood or saliva were rejected and an alternative site chosen. The perio-paper strips were placed in micro-tubes and stored at -80°C until required. Perio-paper strips were eluted in 250 µl ice cold sterile distilled water before analysis.

MMP-8 activity assay

Greiner® 96 well black high binding plates (Sigma Aldrich, Dorset) were coated with 100 µl/well MMP-8 capture antibody (Catalogue # MAB3316; Merck Millipore, Watford, UK) at a concentration of 2 µg/ml in 0.05 M carbonate buffer, pH 9.6. The plate was covered and incubated at 4°C overnight. The following day, the contents of the wells were discarded and the wells were washed three times with phosphate buffered saline (PBS, pH 7.4) containing 0.05% (v/v) Tween-20 (PBST). The wells were then blocked with 200 µl of PBST containing 1% (w/v) bovine serum albumin (BSA) at room temperature for 1 h. Following the blocking step, wells were washed three times with PBST and incubated at room temperature for 2 h with 100 µl/well GCF samples or recombinant MMP-8 standard (R&D Systems, Abingdon, UK). Recombinant MMP-8, supplied in its pro-form, was activated (as directed by manufacturer, R&D systems) by pre-treatment with 1 mM 4-aminophenylmercuric acetate (APMA) for 1 h at 37°C, prior to use in the MMP-8 activity assay. All healthy and periodontitis GCF samples, as well as the APMA-activated MMP-8 standards (3.125 - 100 ng/ml), were diluted in AnaSpec MMP assay buffer (AnaSpec, Fremont, CA, USA) prior to analysis in the MMP-8 activity assay. Following the incubation step with GCF samples or standards, plates were washed three times with PBST. 50 µl AnaSpec MMP assay buffer and 50 µl of 10 µM AnaSpec 520 MMP fluorescence resonance energy transfer (FRET) substrate SB-XIV (AnaSpec, Fremont, CA, USA) was then added to each well. Prior to use, the FRET substrate had been reconstituted to 1 mM in dimethyl sulfoxide (DMSO) and diluted to 100 µM in MMP Assay Buffer (AnaSpec, Fremont, CA, USA) before storage in aliquots at -20°C. Fluorescence measurements were recorded immediately at excitation and emission wavelengths of 485 nm and 525 nm, respectively. Measurements were recorded every 5 min over a period of 70 min on a microtitre plate reader (Genios, Tecan, Reading UK) using Magellan software (Tecan Reading UK) and the results were displayed as relative fluorescence units (RFU) per minute (RFU/min). The detection limit of the MMP-8 activity assay was determined by averaging 30 blank sample readings

(MMP assay buffer in place of sample/standard), recorded as outlined above and adding two standard deviations. The detection limit was expressed in RFU/min.

Cross-reactivity studies

The potential cross-reactivity of the MMP-8 activity assay against three different MMPs, namely MMP-1, -2 and -9 (3.125 - 100 ng/ml) was studied using the method outlined above. Fluorescence measurements were recorded as described above and the activity of each of the MMPs was recorded in RFU/min, using the detection limit of the MMP-8 assay as determined above.

Statistical analysis

All statistical analyses were performed using GraphPad PRISM 4.0 software package (San Diego, CA). The Mann-Whitney test was used for between group comparisons. In all cases, $p<0.05$ was taken to represent a statistically significant difference.

Results

An MMP-8 activity assay was developed, in which a specific antibody captured MMP-8 from samples, before activity was measured using a FRET substrate. A schematic representation of the assay is shown in Fig. 1(a). The detection limit for the MMP-8 activity assay was determined to be 8.2 RFU/min. The MMP-8 activity of samples below the detection limit of the system were recorded as zero in subsequent analysis. A standard curve for MMP-8 activity measurements was constructed using MMP-8 standards and is shown in Fig. 1(b). Cross-reactivity studies using recombinant MMP-1, -2 or -9 showed that these MMPs, even at concentrations up to 100 ng/ml, ranged from an average of -1.4 RFU/min to +1.7 RFU/min and were all well below the detection limit of the MMP-8 activity assay (8.2 RFU/min) (Fig 1(c)). By measuring the MMP-8 activity of unactivated standards before and after binding to the MMP-8 capture antibody, we showed that the assay did not cause undesirable activation of the enzyme. Thus, a sensitive and specific assay was developed for the measurement of active MMP-8.

The assay was then tested for its ability to measure active MMP-8 in both healthy and periodontally diseased GCF samples. Of the 15 individual healthy sites tested, only one exhibited MMP-8 activity

above the detection limit of the system (Fig. 2(a)). In contrast, of the 10 diseased sites tested, 7 had MMP-8 activity levels above the detection limit of the system, ranging from 45 ± 1 RFU/min to 178.9 ± 19.4 RFU/min (Fig. 2(b)). The average MMP-8 activity of the healthy GCF samples studied was 3.7 ± 14 RFU/min and the average MMP-8 activity of the periodontitis GCF samples was significantly higher at 78.7 ± 67.8 RFU/min (***$p < 0.0001$) (Fig. 2(c)).

Discussion

MMP-8 is acknowledged to be one of the central biomarkers in the connective tissue breakdown associated with periodontitis (4, 31-33). GCF levels of MMP-8 have previously been shown to be significantly elevated in periodontitis (17) and MMP-8 has been proposed as a candidate diagnostic marker of periodontitis (21, 34, 35). Based on this considerable body of evidence, the present study was designed to address the need for a novel method to specifically determine MMP-8 activity in GCF (25). Antibody-based commercial ELISAs and IFMAs are commonly used to determine MMP levels in a variety of different samples (25-27, 36). A well-recognised limitation of these assays however, is that they rely on MMP epitope recognition but cannot differentiate between latent and active forms, or indeed between inhibitor bound or unbound forms of the enzyme. Therefore the presence of MMPs in GCF, as measured by ELISA-based methods, does not indicate that the enzymes are present in their active form. Indeed, ascertaining the biological and clinical significance of MMPs in periodontal disease destruction is acknowledged to be limited by difficulties in measuring specific MMP activity within a complex mixture of both host and bacterial enzymes (3). The present study addressed and circumvented the limitations associated with ELISA methods *via* the development of a specific MMP-8 activity assay using a combined approach of interaction with specific antibody to capture MMP-8 from GCF, followed by fluorescent substrate cleavage to measure its activity.

In validating the assay, cross-reactivity studies were undertaken to determine the potential for non-specific detection of MMP-1, -2 or -9 recombinant enzymes. Given that MMP-9 has a common source from neutrophil granules and together with MMP-8 is involved in the connective tissue destruction associated with periodontal disease (7, 17, 25), the lack of detection of MMP-9 in the novel activity assay was particularly relevant for the analysis of GCF samples. This indicated that the antibody capture step was selective in removing only MMP-8 from the GCF samples, thus allowing its specific quantification by the fluorescent substrate.

Roles for several MMPs in periodontal health and disease have previously been proposed (37-42). However, a specific role for MMP-8 in periodontitis can be traced back to studies carried out in the 1980's (43, 44). In later years, using combinations of zymography, ELISA and colorimetric assays, GCF levels of MMP-8 were shown to be significantly higher in chronic periodontitis patients compared with healthy subjects and the levels decreased following treatment intervention (2, 45-47). Furthermore, GCF MMP-8 levels were shown to correlate with disease severity (48, 49), strengthening the rationale for the use of a specific MMP-8 activity assay.

Conclusion

This study is the first to capture active MMP-8 from GCF, and subsequently determine its activity using a fluorescent substrate. We also show for the first time that the activity of MMP-8 was significantly increased in GCF from periodontitis sites compared with healthy sites. The activity assay was shown to be sensitive and specific for MMP-8 and has the major advantage that only the enzymatically active form of MMP-8 will cleave the FRET substrate leading to its specific quantification in the assay. Since it is the active form of MMP-8 that initiates collagen destruction then the ability to measure this activity in GCF should improve our understanding of the role of MMP-8 in health and disease and in due course facilitate biomarker development for periodontal tissue destruction.

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Fig. 1. Development of the MMP-8 activity assay. (a) Schematic representation of the Specific MMP-8 Activity Assay which was developed. Briefly, the wells of a black microtitre plate coated with an MMP-8 specific antibody bind the enzyme in GCF samples and upon incubation with the FRET peptide substrate, active MMP-8 cleaves the substrate, separating the quencher (Q) and fluorophore (F) fragments, thus allowing fluorescence detection. (b) The activity of APMA-activated recombinant MMP-8 standard, in the range 3.125 – 100 ng/ml, in relative fluorescence units (RFU) per min (RFU/min), as determined *via* a specific MMP-8 activity assay (mean ± SD, n=2). (c) Cross-reactivity studies demonstrated negligible detection of MMP-1, -2 or -9 activity in the MMP-8 activity assay, at the three highest concentrations of recombinant standards tested (25, 50 and 100 ng/ml) (mean ± SD, n=3).

Fig. 2. MMP-8 activity measurements in GCF samples. (a) MMP-8 activity in individual healthy (h) GCF samples; (b) individual periodontitis (p) GCF samples and (c) average MMP-8 activity of GCF samples from healthy and periodontitis sites, (mean ± SD, n=2 measurements for each sample; ***$p < 0.0001$).

Supporting Information

Additional Supporting Information may be found in the online version of this article (Fig. S1).

Fig. S1. Control experiment to determine whether latent recombinant MMP-8 standards (in the range 25 – 3.125 ng/ml) could be activated by exposure to the MMP-8 capture antibody.

No Ab: Latent MMP-8 standards not exposed to the MMP-8 capture antibody. *Ab:* Latent MMP-8 standards exposed to the MMP-8 capture antibody (mean +/- SD, n = 2 measurements for each standard).

Results indicate that the basal activity level of the MMP-8 standards post-exposure to the capture antibody were in fact slightly lower than those recorded in the assay lacking the antibody capture step. Thus, binding of latent MMP-8 standard to capture antibody does not lead to its activation and subsequent substrate turnover.
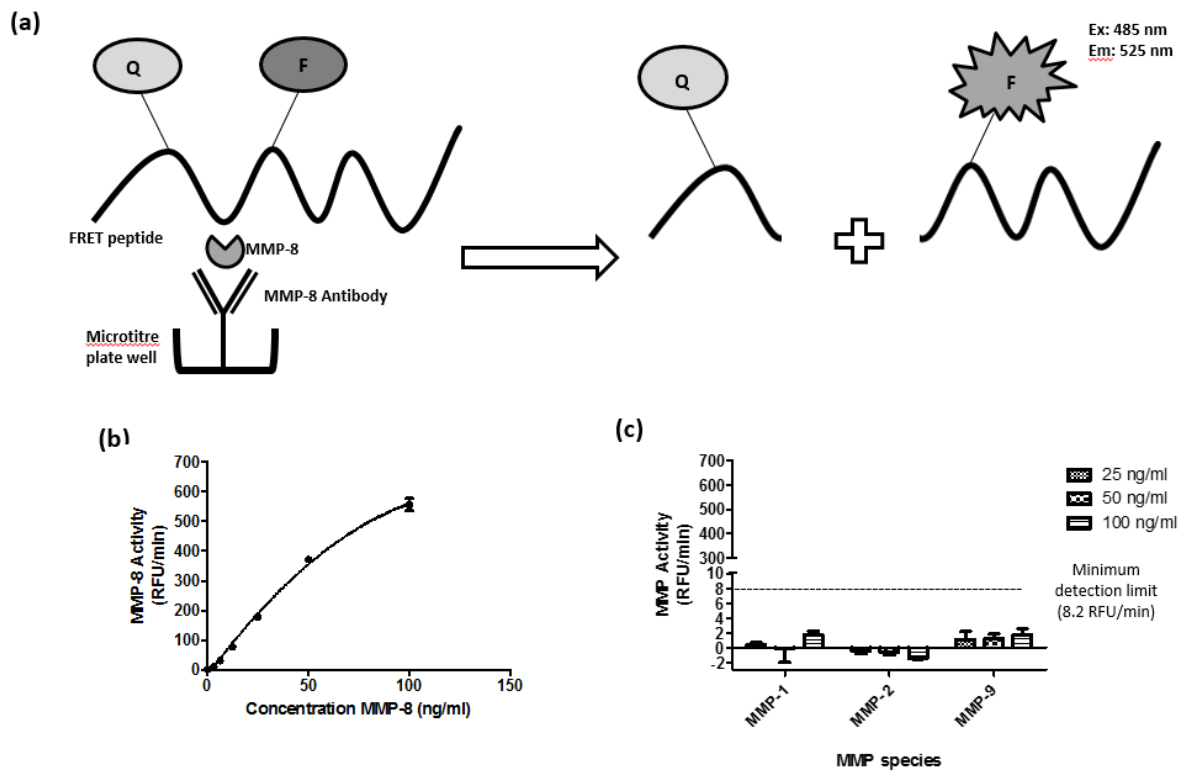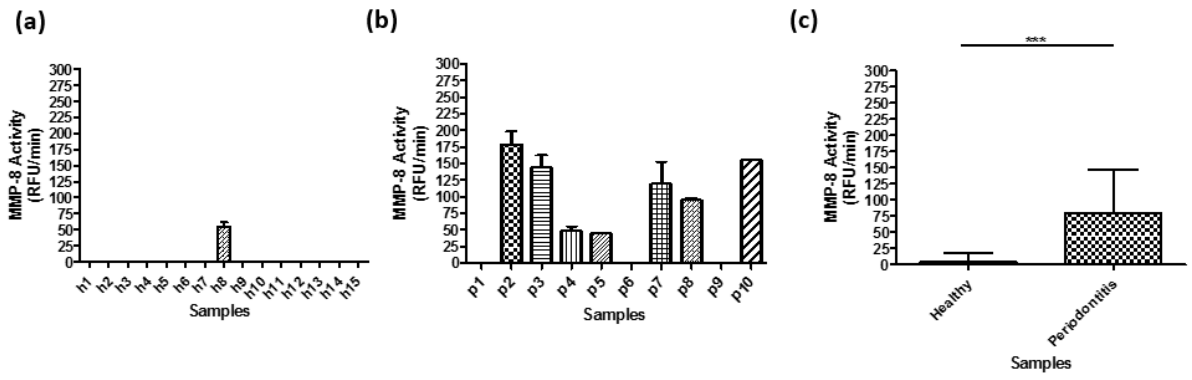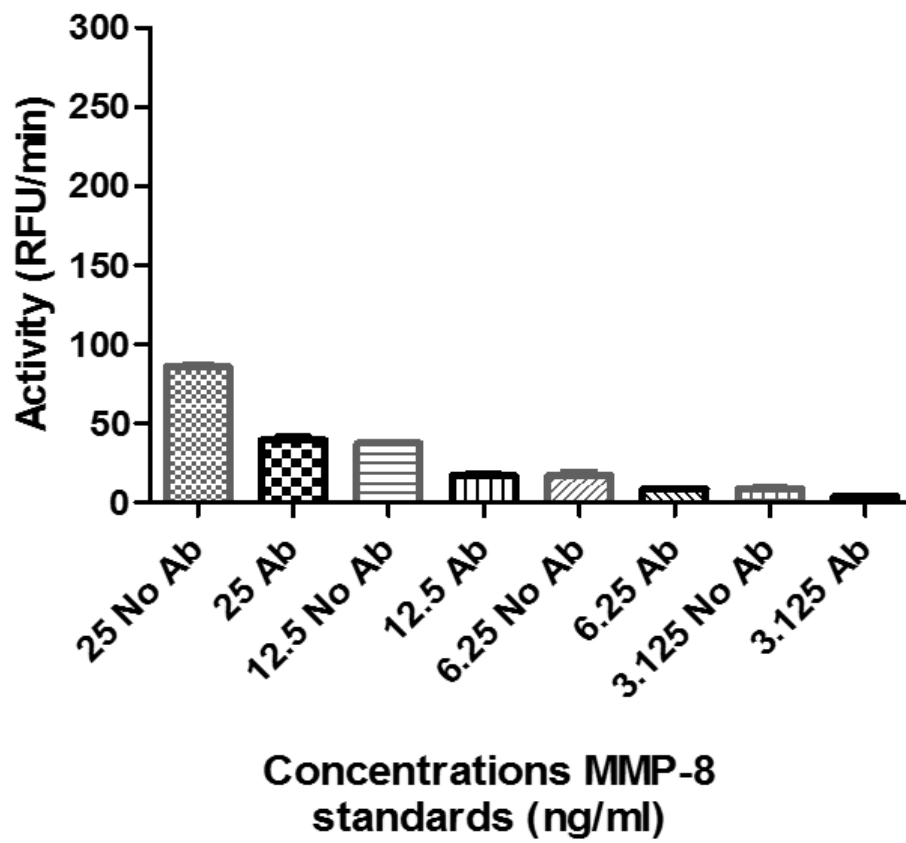
Fig. 1.

(a)

(b)

(c)

Fig. 2.

Fig. S1.