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Experimental tooth pain elevates substance P and matrix metalloproteinase-8 levels in human gingival crevice fluid

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Abstract

Objective. Tooth pain can induce a neurogenic inflammatory reaction in gingiva in association with local elevations of matrix metalloproteinase (MMP)-8, which is considered the major tissue destructive protease in gingival crevice fluid (GCF). The pro-inflammatory neuropeptides released by sensory nerves coordinate the activities of the immuno-effector cells and may influence the secretion of MMP-8. With this background, we studied whether experimental tooth pain can trigger changes in GCF levels of the neuropeptide substance P (SP) and MMP-8. ***Material and methods***. The GCF SP levels of stimulated and non-stimulated teeth were analyzed for SP using a competitive enzyme immunoassay (EIA). The GCF MMP-8 levels were determined by quantitative immunofluorometric assay (IFMA). ***Results***. Painful stimulation of the upper central incisor caused significant elevations in GCF SP and MMP-8 levels of the stimulated tooth. At the same time, the GCF SP and MMP-8 levels of non-stimulated control teeth were unchanged. ***Conclusions***. These data indicate that experimental tooth pain can induce local elevations of SP and MMP-8 levels in GCF simultaneously. This supports the possibility of a local neurogenic spread of inflammatory reactions from intrapulpal to surrounding periodontal tissues.

Key Words: *Gingival crevice fluid, matrix metalloproteinase-8, neurogenic inflammation, pulpal pain, substance P*

Introduction

Painful tooth stimulation and chemical stimulation of gingival nociceptive afferents can induce similar inflammatory reactions in gingivomucosal tissues [1–4]. This process is based on the release of inflammatory mediators from the peripheral nerve terminals, and is thus called axon reflex-mediated neurogenic inflammation [5]. These neurogenic mechanisms may be responsible not only for painful tooth stimulation evoked blood flow elevations in gingiva [3], but also for neuropeptide level elevations in gingival crevice fluid (GCF) of the painful teeth with pulpitis [6]. Matrix metalloproteinases (MMPs) are produced by various immuno-effector cells such as neutrophils, monocytes/macrophages and fibroblast-like cells [7] often influenced by the pro-inflammatory neuropeptides released from the closely apposed sensory nerves in various tissues [8] including gingiva [9]. Interestingly, pulpal pain has been shown to be associated with elevated GCF levels of MMP-8 [10], which is the major tissue-modulating protease, especially in the periodontitis-affected gingiva and GCF [11–14].

With this background, we conducted a pilot study to investigate whether painful tooth stimulation can induce simultaneous changes in GCF SP and MMP-8 levels in humans. To evaluate the spatial spread of pulpal pain evoked effects, we analyzed the SP and MMP-8 levels in GCF collected from the crevices of the non-stimulated lower incisors.

Material and methods

Subjects

Six trained volunteers (4 M, 2 F), ranging in age from 23 to 44 years, were tested in the experiments

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in our study. The subjects were healthy graduate students or researchers who had excellent oral and general health and were free of clinical signs of infection or inflammation in their oral tissues. Informed consent was obtained from each subject prior to the experiments, in accordance with the ethics guidelines of the World Medical Association Declaration of Helsinki (version, 2002). The Ethics Committee of the Medical Faculty of the University of Helsinki approved the study protocol.

Stimulation techniques

Dental stimulation was generated with a constant-current tooth stimulator as previously described [15]. During the experimental sessions, the permanent upper right central incisor (tooth 11) was electrically stimulated at an intensity of three times the individual threshold. The stimulation period lasted 90 s. The stimulator featured a built-in circuit for measuring electrode resistance. The resistances of the stimulated teeth were monitored throughout the experiment in monopolar coupling, and they ranged from 1.5 to 3.5 MΩ.

GCF samples

GCF samples were collected from the mesiobuccal and distobuccal aspects of each tooth prior to tooth stimulation, during stimulation, and after stimulation ended, as previously described [10]. Briefly, gingival tissue around the stimulated and non-stimulated control teeth underwent careful periodontal and panoramic radiographic examination. None of the volunteers had used antibiotics within the preceding month. To avoid blood contamination and possible stimulation of GCF flow during clinical measurements, GCF samples were collected prior to other clinical recordings. The sampling site was isolated with cotton rolls and dried with a gentle air stream; GCF was then collected using standard filter paper strips. The strip was placed within the crevice until mild resistance was felt, and left there for 30 s. After that, the strip was immediately placed in polypropylene tubes. Thereafter, each strip was eluted into 300 μl of 20 $mmol \cdot l^{-1}$ phosphate buffer pH 6.0, containing 0.15 $mol \cdot l^{-1}$ NaCl and 0.1% Tween 20 [16]. The eluates were stored at $-70^{\circ}C$ prior to analysis.

Substance P immunoassay (EIA)

We measured the substance P in GCF from the stimulated and control teeth using a competitive enzyme immunoassay (EIA) kit (R&D Systems Inc., Minn., MN, USA) as described earlier [17–20]. Results were expressed as $ng \cdot l^{-1}$.

Immunofluorometric assay (IFMA) for MMP-8

We analyzed MMP-8 concentrations in the GCF by a time-resolved immunofluorometric assay (IFMA); the amounts of monoclonal antibodies 8708 and 8706 for MMP-8 were 1.5 μg and 0.5 μg per assay, respectively [13,16,21]. Results were expressed as $\mu g \cdot l^{-1}$.

Course of the experiments

The investigation comprised two sessions with a 1-week interval, as described earlier [10]. In the first session, six human volunteers underwent painful tooth pulp stimulation ($3 \times$ pain threshold) of the upper right central incisor. The subjective pain levels were evaluated using a visual analog scale (VAS) (0 = no pain, 100 = the worst imaginable pain intensity). A total of eight GCF samples were collected from the upper right central incisor (tooth 11 = stimulated tooth) and from the non-stimulated lower right central incisor (tooth 41). Samples one and two were taken 2 and 1 min before the stimulation began and their average value served as a baseline. Samples three and four were taken 30 s and 1 min after stimulation began, and their average value served as a stimulation level. Samples 5–8 were taken 4 and 8 min after the end of tooth stimulation. In the second control session, GCF samples were similarly collected without tooth stimulation from six subjects who had participated in the first session. During tests, each subject's heart rate (HR) and mean arterial blood pressure (MAP) responses were recorded semi-automatically (Omron digital blood pressure monitor, HEM-705C; Osaka, Japan) from the left upper arm at the same time as the GCF samples were taken. HR and MAP measurements served to clarify whether pulpal pain could have induced some systemic stress reactions as indicated by possible changes in cardiovascular parameters [22].

Data analysis

SP and MMP-8 values were normalized due to large inter-individual variability. The grand mean of all SP and MMP-8 values from each measurement site was calculated separately. For ongoing statistical analysis, the means of baseline, stimulation, 4 min after and 8 min after the end of stimulation values were extracted and normalized with this grand mean (percent change as compared with grand mean). A Friedman non-parametric ANOVA was used to compare baseline, stimulation, and post-stimulation periods for MMP-8 values and SP values, respectively. In the event of significance, a Wilcoxon's matched pairs test was performed for post hoc comparisons of baseline with stimulation and with post-stimulation periods. $P < 0.05$ was considered significant.

Results

Clinical and radiographic evaluation of sites exposed to painful stimulation and sites used as control demonstrated that gingival and periodontal health was excellent. During experiments, painful tooth pulp stimulation had no effect on GCF flow at any site (not shown).

The mean (SD) intensity of tooth stimulation was 45 (16.1) μA, while the respective average pain magnitude estimate on VAS scores was 67.5 (15.4).

Stimulation of tooth 11 increased the SP and MMP-8 levels in the adjacent GCF (Table I). For SP, the significant effect ($p = 0.03$) was found only during the stimulation period. For MMP-8, the levels remained elevated 4 min ($p = 0.01$) and 8 min ($p = 0.04$) after the end of stimulation. Simultaneously, no marked changes in GCF SP or MMP-8 levels could be detected at tooth 41 (data for MMP-8 not shown). The control session without stimulation showed that the repeated measurements did not modulate GCF SP or MMP-8 levels (data not shown). Tooth stimulation had no marked effects on systemic HR or MAP values.

Discussion

In the present study, high intensity stimulation of the upper right incisor induced a local elevation in the GCF SP and MMP-8 levels of the stimulated tooth. In contrast, the GCF SP and MMP-8 levels of the ipsilateral lower incisor remained unchanged. These results indicate that painful tooth stimulation can induce local neurogenic inflammatory responses that can enhance proteolytic or tissue-modulating potential in the very adjacent gingival tissues, and that pulpal pain can contribute to the local regulation of SP and MMP-8 levels in GCF.

At local sites of various tissues, the sensory nerves are closely apposed to immuno-effector cells, which are known to produce MMPs [7,8,23]. Clinically, the pro-inflammatory neuropeptides released by sensory nerves coordinate the responses of the immuno-effector cells [8], and may have an important role in the pathogenesis of periodontitis [9,24]. Studies on animals have shown the existence of branched nerves innervating both intrapulpal and periodontal tissues [25]. Activation of nociceptive afferents induces a local axon reflex-mediated neurogenic inflammatory reaction [26] which results from the release of pro-inflammatory neuropeptides such as SP and calcitonin-gene-related-peptide (CGRP) from the peripheral nerve terminals. Interestingly, recent clinical findings in patients suffering from pulpal pain and inflammation confirm that pulpal pain can cause an axon reflex-mediated elevation of these pro-inflammatory mediators in the GCF of the painful tooth [27]. Thus, the present pulpal stimulation-evoked increase in the GCF SP and MMP-8 levels of the stimulated tooth could result from a local neurogenic, possibly axon reflex-mediated, inflammatory mechanism.

The release of neuropeptides from the peripheral terminals of afferent nerve fibers can modulate inflammatory mediators, cytokines and proteolytic enzymes in inflammatory diseases [28]. Interleukin (Il)-1, tumor necrosis factor (TNF)-α and prostaglandin E_2 (PGE_2) can trigger neutrophils, gingival fibroblasts and epithelial cells to express MMPs including MMP-8 [7]. SP and related neuropeptides exert extensive pro-inflammatory actions such as increased capillary permeability, vasodilatation, PGE_2, MMPs, Il-1 and TNF-α secretion by resident cells at the sites of inflammation [28]. Overall, neuropeptides, either directly or through action on cytokines, prostaglandins, and TNF-α, can induce the secretion of MMP-8 in the periodontium [7,21,28,29]. In our study, pulpal pain elevated GCF SP levels during stimulation, whereas maximal elevations in GCF MMP-8 levels were found 4 min after the stimulation period ended. According to these findings it is possible that pulpal pain first evoked SP release, which triggered the following MMP-8 elevations in the present investigation.

The resistances of stimulated teeth varied between subjects from 1.5 to 3.5 MΩ in the present investigation, indicating that no short-circuiting to periodontal tissues occurred [30]. Moreover, electrophysiological evidence indicates that the maximal strength (below 60 μA) of the electrical current used in this study for

Table I. Pulpal pain of tooth 11 raised local GCF SP and MMP-8 levels[a] but had no effect on GCF SP levels[a] of the control tooth (tooth 41) or on systemic HR[b] or MAP[c]. All data presented as mean values (SD) over all subjects ($n = 6$)

			After end of stimulation	
	Baseline	Stimulation	4 min	8 min
GCF SP of the stimulated tooth ($ng \cdot l^{-1}$)	3.42 (3.18)	4.51 (4.41)*	4.39 (4.41)	3.68 (3.67)
MMP-8 of the stimulated tooth ($\mu g \cdot l^{-1}$)	23.2 (20.08)	47.2 (40.91)*	57.1 (49.23)*	42.9 (45.80)*
GCF SP of the control tooth ($ng \cdot l^{-1}$)	3.72 (2.57)	3.60 (2.93)	3.65 (3.18)	3.71 (3.06)
Heart rate ($beat \cdot min^{-1}$)	67 (7.83)	65 (6.12)	65 (5.63)	66 (8.81)
Mean arterial pressure (mmHg)	92.4 (4.65)	93.4 (5.63)	91.6 (4.41)	91.9 (4.65)

[a]Quantitative GCF SP concentration based on EIA and GCF MMP-8 on IFMA data.
[b]Heart rate (HR).
[c]Mean arterial pressure (MAP). An asterisk (*) indicates the significant difference compared with baseline (Wilcoxon matched pairs).

dental stimulation, even when applied to periodontium, does not activate extrapulpal nerve fibers [30]. Earlier research [31] with electrical tooth stimulation has shown that the stimulation evoked sensation is due to activation of intradental nerve fibers if it is obtained below 150 μA. These findings indicate that it was probably the activation of intrapulpal nociceptive afferent fibers that triggered SP and MMP-8 changes in GCF.

Clinical studies have shown that SP [27] and MMP-8 [32] are more abundant in pulp tissues from painful human teeth compared with healthy. Moreover, tooth pain in symptomatic pulpitis [6], or caused by orthodontic tooth movement [33], is associated with elevated GCF SP levels of these teeth. On the other hand, tooth pain induced by orthodontic tooth displacement may also increase GCF MMP-8 concentrations [16]. These clinical findings indicate that painful teeth may be allied with elevated levels of pro-inflammatory SP and MMP-8 both in pulp tissue and in adjacent GCF.

Pulpal pain and inflammation lead to an increase in intrapulpal pressure [34]. Thus, increased fluid flow from the root canal system through the apical foramen and dentinal tubules may cause delivery of SP and MMP-8 to the periodontal ligament space. In our study, the subjects' gingival health was excellent and without gingival recessions, which contradicts the notion that increased GCF SP and MMP-8 levels resulted from direct migration from tooth pulp to GCF via dentinal tubules. It is also possible that neuropeptides and MMPs in GCF migrated from the dental pulp through the apical foramen, but the long diffusion pathway, which markedly reduces concentrations of the diffuses [35], would tend to disprove this hypothesis.

Stress has been shown to modulate some periodontitis-relevant immune parameters in GCF [36] and also SP levels in peripheral blood flow. Since pulpal pain evoked only local elevations in GCF SP and MMP-8 levels and caused no marked modulations in systemic cardiovascular parameters, a systemic stress mechanism probably did not significantly contribute to the present results.

The present investigation indicates that experimental pulpal pain can produce local elevations in GCF levels of the pro-inflammatory neuropeptide SP and a potent host-tissue destructive protease, MMP-8. These data support the possibility of local neurogenic spread of inflammation from intrapulpal to surrounding periodontal tissues. Moreover, this pulp-based neurogenic process may predispose such sites to the progression of periodontal destruction [14]. Alternatively, depending on the role of MMP-8 due its ability to modulate anti-inflammatory cytokines and chemokines, this reaction may also be, at least in part, anti-inflammatory or defensive [37,38].

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