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PAIN-EVOKED ALTERATIONS ON GINGIVAL BLOOD FLOW AND GINGIVAL CREVICULAR FLUID (GCF) NEUROPEPTIDE SP AND COLLAGENASE-2 (MMP-8) LEVELS

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ABSTRACT

Previous studies have demonstrated that clinical pulpal pain can induce the expression of pro-inflammatory neuropeptides in the adjacent gingival crevice fluid (GCF). Vasoactive agents such as substance P (SP) are known to contribute to the inflammatory type of pain and are associated with increased blood flow. More recent animal studies have shown that application of capsaicin on alveolar mucosa provokes pain and neurogenic vasodilatation in the adjacent gingiva. Pain-associated inflammatory reactions may initiate expression of several pro- and anti-inflammatory mediators. Collagenase-2 (MMP-8) has been considered to be the major destructive protease, especially in the periodontitis-affected gingival crevice fluid (GCF). MMP-8 originates mostly from neutrophil leukocytes, the first line of defence cells that exist abundantly in GCF, especially in inflammation. With this background, we wished to clarify the spatial extensions and differences between tooth-pain stimulation and capsaicin-induced neurogenic vasodilatation in human gingiva. Experiments were carried out to study whether tooth stimulation and capsaicin stimulation of alveolar mucosa would induce changes in GCF MMP-8 levels and whether tooth stimulation would release neuropeptide SP in GCF. The experiments were carried out on healthy human volunteers. During the experiments, moderate and high intensity painful tooth stimulation was performed by a constant current tooth stimulator. Moderate tooth stimulation activates A-delta fibres, while high stimulation also activates C-fibres. Painful stimulation of the gingiva was achieved by topical application of capsaicin-moistened filter paper on the mucosal surface. Capsaicin is known to activate selectively nociceptive C-fibres of stimulated tissue. Pain-evoked vasoactive changes in gingivomucosal tissues were mapped by laser Doppler imaging (LDI), which is a sophisticated and non-invasive method for studying e.g. spatial and temporal characteristics of pain- and inflammation-evoked blood flow changes in gingivomucosal tissues. Pain-evoked release of MMP-8 in GCF samples was studied by immunofluorometric assay (IFMA) and Western immunoblotting. The SP levels in GCF were analysed by Enzyme immunoassay (EIA). During the experiments, subjective stimulus-evoked pain responses were determined by a visual analogue pain scale. Unilateral stimulation of alveolar mucosa and attached gingiva by capsaicin evoked a distinct neurogenic vasodilatation in the ipsilateral gingiva, which attenuated rapidly at the midline. Capsaicin stimulation of alveolar mucosa provoked clear inflammatory reactions. In contrast to capsaicin stimuli, tooth stimulation produced symmetrical vasodilatations bilaterally in the gingiva. The ipsilateral responses were significantly smaller during tooth stimulation than during capsaicin stimuli. The current finding – that tooth stimulation evokes bilateral vasodilatation while capsaicin stimulation of the gingiva mainly produces unilateral vasodilatation – emphasises the usefulness of LDI in clarifying spatial features of neurogenic vasoactive changes in the intra-oral tissues. Capsaicin stimulation of the alveolar mucosa induced significant elevations in MMP-8 levels and activation in GCF of the adjacent teeth. During the experiments, no marked changes occurred in MMP-8 levels in the GCF of distantly located teeth. Painful stimulation of the upper incisor provoked elevations in GCF MMP-8 and SP levels of the stimulated tooth. The GCF MMP-8 and SP levels of the non-stimulated teeth were not changed. These results suggest that capsaicin-induced inflammatory reactions in gingivomucosal tissues do not cross the midline in the anterior maxilla. The enhanced reaction found during stimulation of alveolar mucosa indicates that alveolar mucosa is more sensitive to chemical irritants than the attached gingiva. Analysis of these data suggests that capsaicin-evoked neurogenic inflammation in the gingiva can trigger the expression and activation of MMP-8 in GCF of the adjacent teeth. In this study, it is concluded that experimental tooth pain at C-fibre intensity can induce local elevations in MMP-8 and SP levels in GCF. Depending on the role of MMP-8 in inflammation, in addition to surrogated tissue destruction, the elevated MMP-8 in GCF may also reflect accelerated local defensive and anti-inflammatory reactions.

Key words: blood flow, Capsaicin, gingiva, gingival crevice fluid, laser Doppler imaging, matrix metalloproteinase-8, neurogenic inflammation, pulpal pain, substance P (SP)

Nina-Li Avellán. Kivun aiheuttamat muutokset ikenen verivirtauksessa sekä ientaskunesteen neuropeptidi SP- ja matriksin metalloproteinaasimäärissä. Purentafysiologian oppiala. Hammaslääketieteen laitos. Turun yliopisto. Annales Universitatis Turkuensis. Sarja-Ser. D Medica-Odontologica 2008.

TIIVISTELMÄ

Tulehdusreaktioon liittyy kipua, verivirtausmuutoksia ja kudostuhoa. Verivirtausmuutokset perustuvat tulehduksen neurogeenisiin vaikutuksiin, joissa mediaattorina toimii kipuhermoista vapautuva neuropeptidi SP. Kudostuhoa aiheuttavat kollagenaasi-entsyymit, joista parodontiumissa kollagenaasi-2:a (MMP-8) pidetään keskeisimpänä. Tämän tutkimuksen tarkoituksena oli selvittää kokeellisesti hampaan ja parodontiumin tulehdusreaktion aiheuttamia muutoksia ienkudoksen verivirtauksessa sekä ientaskunesteen MMP-8 ja neuropeptidi SP määrässä.

Kliininen tutkimus suoritettiin Helsingin yliopiston Hammaslääketieteen laitoksella, Biomedicumissa, Turun yliopiston Hammaslääketieteen laitoksella sekä Erlangenin yliopistossa Saksassa. Tutkimuksessa oli mukana terveitä koehenkilöitä. Kokeellinen tulehdusreaktio aiheutettiin hampaan sähköärsytyksellä ja ikenen capsaicin penslauksella. Ientaskuneste kerättiin standardoidulla stripsi-menetelmällä. Ientaskunesteestä määritettiin tulehdusreaktion aiheuttamat MMP-8- ja neuropeptidi SP-tasojen muutokset. Hammasta ympäröivän tukikudoksen verivirtausmuutokset kartoitettiin non-invasiivisesti laser Doppler imaging-laitteistolla.

Tutkimus osoittaa, että capsaicinin aiheuttama paikallinen hermovälitteinen tulehdusreaktio ikenessä ja limakalvolla ei ylitä yläleuan keskilinjaa. Capsaicin laukaisee myös MMP-8:n vapautumisen läheisten hampaiden ientaskunesteessä, mutta tämä MMP-8 tason nousu ei myöskään jatku yli keskiviivan viereiseen yläleuan puoliskoon. Hermovälitteisen tulehdusreaktion suurempi voimakkuus ärsytettäessä limakalvoa kuin ärsytettäessä kiinnittynyttä ientä tukee hypoteesia, että suun limakalvo on kiinnittynyttä ientä herkempi kemikaalien ja bakteereiden aiheuttamille tulehdusreaktioille. Tutkimustulosten mukaan kokeellinen hammaskipu aiheuttaa paikallisesti ientaskunesteessä MMP-8- ja neuropeptidi SP-tason nousua. Tulokset tukevat hypoteesia, että tulehdusreaktio voi levitä hermovälitteisesti hammasytimestä sitä ympäröiviin kiinnityskudoksiin.

Tutkimus on kliinisesti merkittävä, koska se selvittää tulehdusreaktion neurogeenisiä mekanismeja sekä avain-välittäjäaineiden (neuropeptidi SP ja MMP-8) toiminnan tulehdusreaktion etenemisessä hampaan ja sitä ympäröivän tukikudoksen välillä. Tutkimustulokset luovat perustan tarkemmalle intraoraalisten tulehdustilojen diagnostiikalle sekä uusien täsmällisten hoitomuotojen kehittämiselle.

Avainsanat: capsaicin, hammaskipu, hermovälitteinen tulehdusreaktio, ien, ientaskuneste, laser Doppler imaging, matriksin metalloproteinaasi-8, neuropeptidi SP, verivirtaus

CONTENTS

ABBREVIATIONS

α2-MG	alpha-2-macroglobulin
ANS	autonomic nervous system
BM	basement membrane
CNS	central nervous system
C-terminus	carboxyl terminus
ECM	extracellular matrix
EDTA	ethylenediamine tetra-acetic acid
EIA	enzyme immunoassay
GCF	gingival crevice fluid
HR	heart rate
IFMA	time-resolved immunofluorometric assay
IL-1β	interleukin1β
kDa	kilodalton
LDF	laser Doppler flowmetry
LDI	laser Doppler imaging
LPS	lipopolysaccharide
MAP	mean arterial blood pressure
MT-MMP	membrane-type MMP
MMP	matrix metalloproteinase
MMP-8	collagenase-2/ neutrophil collagenase
N-terminal	amino terminal
PGE_2	prostaglandin E_2
PMN	polymorphonuclear leukocyte, neutrophil leukocyte
RF	reticular formation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SP	substance P
TIMP	tissue inhibitor of matrix metalloproteinase
TNF-α	tumour necrosis factor-α
TTBS	10 mM Tris-HCl, pH 7.5, 0.05% Triton X-100, 0.2 M NaCl
VAS	visual analogue scale
Zn	zinc

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications referred to in the text by their Roman numerals.

I

Kemppainen P, Avellán N-L, Handwerker H.O, Forster C.
Differences between tooth stimulation and capsaicin-induced neurogenic vasodilatation in human gingiva. Journal of Dental Research 2003; 82(4): 303-307.

II

Avellán N-L, Sorsa T, Tervahartiala T, Mäntylä P, Forster C, Kemppainen P.
Painful tooth stimulation elevates matrix metalloproteinase-8 levels locally in human gingival crevicular fluid. Journal of Dental Research 2005; 84(4): 335-339.

III

Avellán N-L, Kemppainen P, Tervahartiala T, Vilppola P, Forster C, Sorsa T.
Capsaicin-induced local elevations in collagenase-2 (matrix metalloproteinase-8) levels in human gingival crevice fluid. Journal of Periodontal Research 2006; 41: 33-38.

IV

Avellán N-L, Sorsa T, Tervahartiala T, Forster C, Kemppainen P.
Experimental tooth pain elevates substance P and matrix metalloproteinase-8 levels in human gingival crevice fluid. Acta Odontologica Scandinavica 2008, in press.

1 INTRODUCTION

Pain and inflammatory reactions are known to be associated with blood flow changes, and, for example, in migraine type of headache, pain is supposed to be vascular in origin (Goadsby 1997). There are also distinct signs of blood flow alterations in other types orofacial pain syndromes such as trigeminus neuralgia and pain-related masticatory dysfunctions (Suvinen *et al.* 2005).

Pain-provoked alterations in blood flow are neuronal, driven via local axon reflexes and centrally mediated somato-autonomic vascular reflexes (Wallin 1990). It is well documented that activation of cutaneous nociceptive afferents induces local vasodilatation (Wårdell *et al.* 1993), which is caused by the release of inflammatory mediators from the peripheral nerve terminals, and thus called axonreflex-mediated neurogenic inflammation (Holzer 1988). Unmyelinated nociceptive C-fibres, containing vasoactive agents such as substance P (SP) and calcitonin-gene-related peptide (CGRP), are mainly responsible for this inflammatory type of pain and associated blood flow increases (Holzer 1988). These vasoactive neuropeptides are released upon activation of C-afferent fibres leading to a local blood flow increase. Axon reflex vasodilatation usually spreads symmetrically around the nociceptive stimulus and corresponds to the size of the receptive fields of the stimulated nociceptive afferents (Wårdell *et al.* 1993). Interestingly, several recent studies in humans have demonstrated that especially orofacial C-fibre mediated pain (for example tooth pain) can evoke not only local axon reflexes but also centrally mediated blood flow alterations in various trigeminal regions (Kemppainen *et al.* 1994, 2001a, b) and even central pain-sensitive structures (May & Goadsby 1999).

There are two types of afferent nerve fibres, A-delta and C-fibres, within the tooth pulp (Närhi *et al.* 1996). Electrical tooth stimulation (pulse duration 10 ms, frequency 5 Hz) at pain threshold level activates selectively A-delta fibres, and by increasing stimulus intensity it is possible to activate also pulpal C-fibres (Närhi 1985; Virtanen 1985). In the present series of studies, we designed experiments to determine whether tooth stimulation would evoke MMP-8 levels in GCF. The present study was also conducted to clarify whether there are differences between tooth stimulation current and different pulpal nerve fibre population and stimulation-evoked MMP-8 levels in GCF. The preliminary findings indicated that only high intensity tooth stimulation, capable of activating pulpal C-fibres, induced elevations in GCF MMP-8 levels. Therefore, 3x the individual pain threshold was used (average current intensity 47 μA) in this study.

In the present study, the laser Doppler imaging (LDI) technique was applied for the first time to record blood flow in human gingiva. Although it is an indirect measure, LDI has previously been shown to be a successful method for the documentation of orofacial blood flow changes (Kemppainen *et al.* 2001a, b). In contrast to laser Doppler flowmetry (LDF), as used, for example, in the determination of blood flow in healthy (Baab *et al.* 1986) and diseased human gingiva (Baab *et al.* 1990), LDI has the advantage of giving simultaneously information on the spatial distribution of vaoactive changes from separate tissues.

It has been recognised that toxins, enzymes and metabolites of various bacteria of the dental plaque are responsible for the initiation of the inflammation in the periodontium (Listgarten 1987). Recent animal studies (Fazekas *et al.* 1990; Kondo *et al.* 1995) have shown that similar inflammatory reactions in the gingiva can be induced experimentally by activating C-afferent nociceptors in gingivomucosal tissues. It has been suggested that intact function of these nociceptive fibres serves as a primary physiological defence mechanism in periodontal tissues (Kondo *et al.* 1995), the impairment of which can lead to reduced periodontal inflammatory function as found in diabetic rats (Györfi *et al.* 1996). However, to the best of our knowledge no systematic studies on the existence and characteristics of C-fibre mediated neurogenic inflammatory reactions in human gingivomucosal tissues have been performed.

2 REVIEW OF THE LITERATURE

2.1 STRUCTURE AND PHYSIOLOGY OF THE PERIODONTIUM

The periodontium, the supporting structures around the teeth, comprises: 1) the gingiva, 2) the periodontal ligament, 3) the root cementum, and 4) the alveolar bone. The periodontium balances masticatory forces and participates in the defence reaction against microbial infections, chemical and physical factors and irritants.

2.1.1 The gingiva

The gingiva surrounding the teeth consists of two parts: 1) **free gingiva** extends from the cemento-enamel junction to the most coronal soft tissue margin and includes the interdental papillae; 2) **attached gingiva** is coral pink in colour and keratinised. Attached gingiva is situated between the free gingiva and the mucogingival junction and is tightly fastened to the alveolar bone.

In the apical direction the gingiva is continuous with the loose, darker red **alveolar mucosa**, from which the gingiva is separated by a borderline called the mucogingival junction. The non-keratinised alveolar mucosa is loosely bound to the underlying bone.

The gingiva is covered by three different kinds of epithelia: 1) the visible gingiva facing the oral cavity is covered by **oral epithelium**. The oral epithelium and the underlying connective tissue contain lots of blood vessels and nerve endings and have rapid metabolism. The oral epithelium is either keratinised or parakeratinised; 2) the oral epithelium continues as non-keratinised or parakeratinised sulcular epithelium, which faces the tooth and is not bound to the tooth surface. **The sulcular epithelium** and the tooth form the v-shaped gingival crevice, sulcus, which is the most critical area concerning the physiology of the periodontium. It allows bacterial growth in the crevice and may start periodontal diseases; 3) **the junctional epithelium** provides the contact between the tooth and the gingiva and forms the bottom of the gingival sulcus. It is non-keratinised. The epithelial attachment of the junctional epithelium, from the internal basal lamina, is fastened to the enamel via hemidesmosomes. Substance P and CGRP have been found immunohistochemically in nerve fibres supplying the junctional epithelium (Byers *et al.* 1987; Nagata *et al.* 1992). Junctional epithelium has a high cellular turnover rate and wide intercellular spaces allowing inflammatory cell emigration and gingival crevice fluid (GCF) flow.

Underlying the gingival epithelial layer is the connective tissue layer. Type I collagen is the major type of collagen and fibroblasts are the major resident cells in the gingival connective tissue. Connective tissue has rapid metabolism, which allowes the fast recovery of small wounds in the gingiva.

2.1.1.1 Gingival crevice fluid (GCF)

Gingival crevice fluid (GCF) is a mixture of serum origin and acts as a vehicle of important defence mechanisms. After the onset of plaque formation, the permeability of the connective tissue vasculature increases, which can be clinically detected by increased gingival crevice fluid flow. In the healthy gingival crevice GCF is released only in small amounts. Quantification of the GCF volume has been used to reveal the inflammatory status of periodontal tissues. GCF volume increases in the order healthy gingiva, gingivitis, and periodontitis (Nakamura 2000), but the increased flow does not necessarily reflect periodontal disease activity.

The GCF composition has been evidenced to follow and reflect the health and disease of the adjacent gingiva (Cimasoni 1983). Qualification of the collected GCF can be used to reflect the activity of periodontal tissue inflammation. In periodontitis patients, repeatedly elevated GCF MMP-8 concentrations may indicate sites at risk of progression of periodontitis as well as patients with poor response to conventional periodontal treatment (Mäntylä *et al.* 2003).

GCF can be collected by several techniques: with paper filter strips, micropipette tubes using gingival washing and capillary tubing. Harvesting by filter paper strips is the most commonly used method. The collection time can affect the harvested GCF. The composition of GCF can change if the tissue is irritated. The sampling time is usually 30 s or less (Mäntylä *et al.* 2003, 2006), but also 3 min or up to 5 min has been used in GCF studies (Apajalahti *et al.* 2003).

2.1.2 Periodontal ligament

The periodontal ligament connects the tooth to the alveolar bone. The collagen ligaments (principally type I collagen) fill the 0.15-0.4 mm space between the root cementum and the alveolar bone process. The metabolism in the periodontal ligament is even more rapid than in gingival connective tissue. The periodontal ligament is divided into groups depending on its orientation. The principal cells of the periodontal ligament are differentiated fibroblasts. The periodontal ligament acts as a network that forms a defence barrier against mastication forces. The fibroblasts respond to the alterations associated with tooth mobility: connective tissue degradation increases in tissue pressure site where strong masticatory forces are directed, and the construction of the ligament fibres is elevated in stretching site. The periodontal ligament is able to adapt to normal forces and keep the periodontal space standard. Excessive non-physiological forces can cause damage to the periodontal ligament and lead to extension of the periodontal space.

2.1.3 The root cementum

A thin layer of the cementum covers the root of the tooth. **The root cementum** attaches the tooth to surrounding alveolar bone via the periodontal ligament. The root cementum is asymmetrically covered by cementocytes. The thickness of the root cementum is dependent on the masticatory forces: in the pressure site the cement is thinner than in the stretching site. The formation of the cement is linked to the adaptation mechanisms of the periodontium towards the masticatory forces.

2.1.4 The alveolar bone

The alveolar bone is dependent on the existence of the teeth, and the loss of teeth leads to resorption (Araújo & Lindhe 2005). The pressure towards the bone causes the activation of osteoclasts and leads to resorption of the bone, while the stretching forces via the periodontal ligaments activate the osteoblasts to bone formation.

2.2 STRUCTURE AND PHYSIOLOGY OF TEETH

Complete deciduous dentition with 20 teeth and complete permanent dentition with 32 teeth, comprises: 1) enamel, 2) dentine, and 3) dental pulp. The tooth differs from the other organs of the body due to its structure and the exceptionally rich innervation of its pulp.

2.2.1 Enamel

Enamel is semi-translucent. It is grey or bluish-white in colour. Except at the unworn biting edges of the incisors, its colour is modified by that of the underlying dentine, producing the characteristic yellowish-white appearance of the crown. The hardness of enamel is considerable, and this is what allows enamel to withstand masticatory loads and protect the underlying dentine. The modulus of elasticity of enamel is lower than that of dentine just as well is the compliance of enamel lower than that of dentine (Pashley 2002). Enamel is indeed the hardest tissue in the body. In its mature state enamel is highly mineralised, containing by weight 96 per cent inorganic material, 1 per cent organic material and 3 per cent water. The inorganic component of mature enamel is mainly in the form of hydroxyapatite crystals (Berkovitz *et al.* 1978).

2.2.2 Dentine

Dentine forms the bulk of the tooth. In the crown, it is covered by enamel, from which it is demarcated by the amelodentinal junction. In the root it is covered by cementum, the boundary between them

being termed the cemento-dentinal junction. The dentine surrounds the pulp cavity. Dentine is pale yellow in colour, and this is what imparts the colour to the crown of the tooth through the semi-translucent enamel. It is harder than bone and cementum, but much softer than enamel. Because it is traversed by a system of tubules, dentine is considerably more permeable than enamel. On a wet weight basis, dentine is composed of approximately 70 per cent inorganic material, 18 per cent organic material and 12 per cent water. Thus, dentine has a much higher content of organic material than enamel. As in other hard tissues, the principal inorganic component is hydroxyapatite. Most of the organic component is collagen (Berkovitz *et al.* 1978).

2.2.3 Dental pulp

The dental pulp cavity consists of a pulp chamber in the crown from which canal(s) pass down into the root(s). As a general rule, the pulp cavities follow the contours of the teeth. Each root canal opens by a foramen/foramina at the apex of the root. The pulp is continuous with the connective tissue of the periodontal ligament through the apex of each root and commonly lateral or accessory root canals. The components of the pulp are common to all loose connective tissue, comprising cells, fibres, blood vessels and nerves. The fibres of the pulp are principally collagenous. Three types of cells can be recognised in the dental pulp: odontoblast, fibroblast and defence cells. The pulp is composed of approximately 25 per cent organic material and 75 per cent water on a wet weight basis (Berkovitz *et al.* 1978).

2.2.3.1 Blood supply of the tooth pulp

The neurovascular bundle enters the pulp at its apical foramen, which in young teeth is wide and may take the form of a delta with several canals. Arterioles are the largest vessels found in the pulp. The arterioles terminate in a rich, subodontoblastic capillary plexus from which small capillaries pass into the odontoblastic layer. Small venules drain the odontoblast layer and pass obliquely across the pulp chamber to join several small veins in the neurovascular bundle (Berkovitz *et al.* 1978). There are numerous anastomoses between venules and arterioles (Kramer 1960).

2.2.3.2 Nerve supply of the pulpodentin complex

Compared to similar volume of connective tissue elsewhere in the body, the dental pulp is richly innervated. The number of nerve fibres entering the tooth is enormous; for example, the human premolar receives more than 900 sensory afferent nerve fibres (Reader & Foreman 1981). These fibres consist of nociceptive unmyelinated C-fibres (72 per cent) and thinly myelinated A-delta fibres (28 per cent). Regardless of stimulus type (electrical, mechanical, thermal or chemical), the only sensation that can be experienced from the pulp is pain (Anderson *et al.* 1970). The course of the nerves follows

closely that of the blood vessels, terminating in a dense plexus beneath the odontoblasts (Raschkow's plexus) (Berkovitz *et al.* 1978). The number of nerve fibres in the middle third of the pulp is estimated to be three times the number found in the apical area (Holland & Robinson 1983). It has been shown in histological studies that the location of the nerve terminals in dentine is limited to the inner 150-200 µm of the tubules. The outer layers of dentine are not innervated (Byers & Matthews 1981; Byers 1984).

The pulpodentin complex is among the most densely innervated tissues in the body. The profuse innervation of the pulp and dentin contains many neuropeptide-rich fibres that can release peptides when stimulated. The timing, concentration and location of secreted neuropeptides act as important signals for other pulpal cells about the status of the tooth. Neural agents are an important signal for neurogenic inflammation for stimulation and repair (Byers & Närhi 2002).

The vasodilatory functions of sensory innervation in teeth are opposed by vasoconstriction by the sympathetic fibres (Olgart 1996). The sympathetic fibres are much less numerous than are sensory fibres (Fried *et al.* 1988). Sympathetic fibre distribution also differs from that of sensory fibres in that they are located mainly in deeper pulp and along blood vessels. Parasympathetic activity can affect blood flow in teeth, but it is not clear whether that is from intradental or periodontal sites; in any case, the relative importance of parasympathetic activity is much less than that of sympathetic activity (Olgart 1996).

Some afferent nerve fibres may branch to innervate both the dental pulp and the adjacent tissues or multiple teeth. To some extent, such organization may contribute to the poor localization of dental pain and may also allow neurogenic vasodilatation and inflammatory reactions to occur in an area of tissue wider than that affected by the original insult. Correspondingly, within the dental pulp the terminal branching of the nerve fibres may contribute to the spread of inflammatory reactions (Närhi *et al.* 1996).

2.2.3.3 Differences between the nerve supply of the pulpodentin complex and the supporting tissues

The tooth pulp is surrounded by a capsule of hard tissue, and the pulp lacks the receptor structures that are typical of the skin. The fact that pain and the weaker prepain are the only sensations which can be felt by the stimulation of pulpal nerves is in accordance with the notion that the pulp contains only free nerve endings instead of specialised receptor structures. The pulpal nerve fibres have their cell bodies located far away in the trigeminal ganglion for sensory neurons. The apical region of the tooth is supplied with several nerve branches that are distributed both to the pulp and periodontium. The apical region is also innervated by branches coming through the alveolar bone and along the mucous membrane of the mouth (Byers 1984).

The nerve fibres in the pulp and dentin are components of a larger trigeminal peripheral nervous system that also includes sensory innervation of the gingiva, junctional epithelium, periodontal ligament, tongue, lips, mastication muscles, and the temporomandibular joint. Each part of the system contributes different kinds of somatosensory information needed for tooth use and preservation. For example, from the gingiva we perceive sensations of touch, pressure and temperature via activation of special mechanoreceptors or thermoreceptors. The junctional epithelium is richly innervated by sensory fibres that release neuropeptides to regulate vasodilatation and transmigration of leukocytes across the epithelium into the oral cavity in defence against oral pathogens. The periodontal ligament contains a number of Ruffini mechanoreceptors. These mechanoreceptors give us our sensation of tooth touch and occlusal plane during chewing, speech, and swallowing. Some part of the sensory information from the periodontal mechanoreseptors may remain unconscious and subserve automatic responses needed for the regulation of masticatory functions. All the orofacial tissues also have specific and polymodal nociceptive nerve fibres that initiate acute pain sensation if there is damage or inflammation. Together, the multiple nerve fibre systems of these regions provide an integrated regulatory system acting on teeth and their supporting tissues (Byers & Närhi 2002).

In the last few decades, a number of studies have addressed the question of bilateral innervation of the maxillary incisors. There is at present no consensus as to whether the nerves may cross the maxillary midline or not, since some reports (Anderson *et al.* 1977; Avery 1979; Fuller *et al.* 1979; Byers & Matthews 1981; Saag & Reid 1981) have demonstrated electrophysiological and anatomical evidence for such innervation. However, similar studies by some researchers have found little or no evidence of a bilateral supply (Matthews & Lisney 1978; Nord & Rollince 1980; Arvidsson & Gobel 1981; Byers 1984). It is possible that technical problems, such as spread of histological label from the pulp to contralateral tissues, might account for the discrepancy in observations (Sessle 1987).

2.3 PAIN

The taxonomy committee of the International Association for the Study of Pain (Merskey 1979) considered definitions of pain and concluded as follows: "Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage."

2.3.1 Transmission of orofacial nociceptive information to the central nervous system (CNS)

Pain in the orofacial region has a special biological, emotional, and psychological meaning to the patient. Furthermore, apart from headache, which may also involve structures in the mouth and face, acute orofacial pain accompanying acute pathologic states in the teeth and associated structures is probably the most common pain in all the body (Sessle 1987).

2.3.1.1 Nociceptors (Pain receptors)

The peripheral terminals of sensory nerves are characterised by the ability to transduce different types of energy into nerve impulses. Peripheral unmyelinated and finely myelinated axons have in the periphery unmyelinated terminals, which have no specialised receptive structures and are termed free nerve endings. Pain is conducted by two different sets of neurons: thin myelinated A-delta fibres with conduction velocities of 12-30 m/s and neurons with unmyelinated C-fibre axons with conduction velocities of 0.5-2.5 m/s (Guyton & Hall 1996).

Because of this organization, the sensation perceived in response to noxious stimulation consists of two discrete and different components: first sharp and well-localised pain mediated by A-delta fibres, and then delayed, dull pain that is mediated by C-fibres. This C-fibre mediated pain can radiate to a wide area surrounding the affected tissue (Mumford & Bowsher 1976; Närhi 2003). The activation of C-fibres leads to tension of muscles and activation of the autonomic nervous system (Holzer 1988).

2.3.1.2 Trigeminal nerve

The trigeminal nerve (the fifth cranial nerve) is the most significant nerve subserving the orofacial structures. The trigeminal nerve is a mixed nerve containing both sensory and motor fibres (Sessle 2000). The trigeminal nerve is divided into three major divisions: the ophthalmic, the maxillary, and the mandibular. In addition to the trigeminal nerve, the facial nerve (the seventh cranial nerve) with its motor component supplies the muscles of facial expression.
The ophthalmic nerve carries sensory information from the scalp and forehead, the upper eyelid, the conjunctiva and cornea of the eye, the nose (including the tip of the nose), the nasal mucosa, and the frontal sinuses. **The maxillary nerve** carries sensory information from the lower eyelid and cheek, the nares and upper lip, the upper teeth and gums, the nasal mucosa, the palate and roof of the pharynx, the maxillary, ethmoid and sphenoid sinuses. **The mandibular nerve** carries sensory information from the lower lip, the lower teeth and gums, the floor of the mouth, the anterior ⅔ of the tongue, the chin and jaw. The mandibular nerve has both sensory and motor functions comprising the motor innervation of the jaw opening and closing muscles. The areas of cutaneous distribution (dermatomes) of the three branches of the trigeminal nerve have sharp borders with relatively little overlap (unlike dermatomes in the rest of the body, which show considerable overlap).

The three branches converge on **the trigeminal ganglion** (also called **the semilunar ganglion** or **gasserian ganglion**), which contains the cell bodies of afferent sensory nerve fibres. The trigeminal ganglion is analogous to the dorsal root ganglia (also called **the spinal ganglion**) of the spinal cord, which contain the cell bodies of afferent sensory fibres from the rest of the body.

2.3.1.3 The orofacial pain pathways in the CNS

From the trigeminal ganglion, a single large sensory root enters the brainstem at the level of the pons. Immediately adjacent to the sensory root, a smaller motor root emerges from the pons at the same level.

The areas that are reviewed here are the trigeminal nuclear complex, the reticular formation, the thalamus, the limbic structures and the cortex. They are discussed in the order by which neural impulses pass on to the higher centres. Additionally, a few specific comments about innervation and circulation of cranial blood flow are emphasised.

Following activation, the C-fibres and finely myelinated A-delta fibres from orofacial tissue such as dental pulp or gingiva transmit nociceptive signals, primarily via trigeminal nerves, to **the trigeminal nuclear complex** located in the medulla. The sensory complex can be subdivided into the main (principal) sensory nucleus and the spinal tract nucleus. The trigeminal spinal tract nucleus includes the nucleus oralis, nucleus interpolaris, and nucleus caudalis. The subnucleus caudalis extends into the cortical spinal cord and merges with the spinal dorsal horn (Sessle 1987). The nucleus caudalis is not simply a relay station where nociceptive signals are passively transferred to higher brain regions. Rather, this site plays an important role in processing nociceptive signals (Hargreaves 2002). Sessle and colleagues have further proposed that a large proportion of the nociceptive neurones in trigeminal nuclei showing extensive convergence from skin, mucosa, visceral (laryngeal), TMJ, jaw or tongue muscle, tooth pulp, and even neck afferents may underlie the spread and referral of pain which is frequently seen in many craniofacial and intra-oral pain conditions (Sessle 1987).

After the primary sensory afferent neurons synapse in the nucleus caudalis, the interneurons transmit the impulses up to the higher centres, some of them passing through an area called **the reticular formation**. Neurones in each part of the V brainstem complex have axons that may project directly or indirectly, via reticular formation, to the thalamus and are thus implicated as critical elements underlying perceptual as well as emotional and motivational responses to orofacial stimuli (Sessle 1987). The reticular formation controls the overall activity of the brain by either enhancing the impulses on the brain or by inhibiting the impulses (Kalso & Vainio 2004).

At the level of **the thalamus** the sensory information is sent to its specific nuclei. The thalamus is located in the very centre of the brain, and most of the impulses from the lower regions of the brain as well as the spinal cord are relayed through synapses in the thalamus before proceeding to the cerebral cortex (Dionne *et al.* 2006). The thalamus drives the cortex to activity and enables the cortex to communicate with the other regions of the CNS. Current theory states that the areas of

the brain involved in pain processing can be divided into two networks: the lateral pain system, which projects through lateral thalamic nuclei to **the cerebral cortex** including the primary and secondary somatosensory cortex (SI and SII); and the medial pain system, which projects through medial thalamic nuclei to brain regions including the prefrontal and anterior cingulate cortex. The lateral pain system is thought to be responsible for the sensory aspects of pain, such as its location and duration, while the medial pain system is thought to be responsible for the emotional aspects of pain, such as how unpleasant it feels. In the study of Jantsch *et al.* (2005), cortical representation of tooth pain was compared with that of painful mechanical stimulation to the hand. In this study it was concluded that the contralateral SI cortex was activated during painful mechanical stimulation of the hand, whereas tooth pain led to bilateral activation of SI. Thus, there are distinct differences between trigeminal pain and pain from other regions of the body, such as the hand.

The limbic structures function to control our emotional and behavioural activities (Evers *et al.* 1981). Within the limbic structures are centres, the medial pain projection system, which are responsible for specific behaviours, such as anger and rage. The limbic structures also control emotions, such as fear, depression and anxiety. The limbic system is well known to play an important role in pain problems. Modern imaging studies in humans have indicated that the frontal cortical areas get input from the limbic structures (Bantick *et al.* 2002). More recently it has been shown that in frontal cortical regions especially trigeminal pain, e.g. tooth pain, has a greater impact compared to hand stimulation (Jantsch *et al.* 2005). It is concluded that trigeminal pain activates a cortical network which is in several respects different from that activated by painful mechanical stimulation of the rest of the body, not only in the somatotopically organised somatosensory areas but also in parts of the limbic medial pain projection system (Jantsch *et al.* 2005).

The trigeminal nerve innervation supplies a rich network of perivascular fibres to **the cranial circulation**, which contains powerful vasodilator neuropeptides (May & Goadsby 1999). Electrical stimulation of the trigeminal ganglion in both humans and the cat leads to increase in extracerebral blood flow and local release of both CGRP and SP (Edvinsson & Goadsby 1988). Based on more extensive studies in humans it was concluded that the observed dilation of the cerebral vessels in trigeminal pain is not inherent to a specific headache syndrome, but is rather a feature of the trigeminal neural innervation of the cranial circulation. Clinical and animal data suggest that the observed vasodilatation is, in part, an effect of a trigeminoparasympathetic reflex (May & Goadsby 1999).

2.3.1.4 Effect of orofacial painful stimuli on cardiovascular parameters via the autonomic nervous system (ANS)

The autonomic nervous system (ANS) is the part of the peripheral nervous system that controls cardiovascular, digestive and respiratory functions, as well as salivation, perspiration and diameter of the pupils. ANS is separated into two divisions: the sympathetic and the parasympathetic. Sympathetic and parasympathetic divisions typically function in opposition to each other. However, this opposition is better termed complementary in nature rather than antagonistic. As an analogy, one may think of the sympathetic division as the accelerator and the parasympathetic division as the brake. The sympathetic division typically functions with actions requiring quick responses, while the parasympathetic division functions with actions that do not require immediate reaction. Sympathetic can be considered as "fight or flight" and parasympathetic as "rest and digest" (Tracey 2002).

Both sympathetic and parasympathetic fibres have a preganglionic and a postganglionic nerve cell. They meet at a ganglion, where the nerve impulse is transferred from cell to cell, at a synapse, by the chemical transmitter acetylcholine. In parasympathetic fibres, the transmitter in postganglionic nerve fibres is again acetylcholine, while noradrenaline serves as the transmitter in the sympathetic system. Most of the sympathetic nerve filaments that have an effect on blood vessels cause vasoconstriction. The parasympathetic nervous system dilates blood vessels, for example those leading to the GI tract, increasing the blood flow (Nienstedt *et al.* 2004). Noxious stimulation of the orofacial structures has been shown to induce active somato-parasympathetic vasodilatation in the cat face (Izumi & Karita 1992; Shoji 1996).

Nociceptive responses to noxious orofacial stimuli include muscle reflexes and autonomic responses. For example, changes in blood pressure, heart rate, respiration, and sweating have long been recognised as accompaniments of craniofacial pain and have indeed often been utilised as indirect measures of pain (Sessle 1987, 2000).

Pain is able to produce central cardiovascular changes, and, in particular, blood flow changes in various peripheral structures mediated by sympathetic effects in most parts of the skin. However, in part of the face, the blood flow changes are probably mediated by parasympathetic nerves (Drummond 1995; Kemppainen *et al.* 2001a). In parasympathetic nerves, acetylcholine often co-exists with vasoactive intestinal peptide (VIP), which is one of the body's most potent vasodilator substances with a long half-life (Brayden 1987). There is evidence that orofacial organs like the tongue, lip and submandibular glands are innervated by both cholinergic and VIP-ergic parasympathetic nerves (Lundberg 1981, 1982; Kaji *et al.* 1988).

2.4 DISEASED PERIODONTIUM

Chronic periodontal inflammation is characterised by increased irreversible degradation of periodontal ligament collagen fibres leading to loss of tooth attachment and apical and lateral migration of gingival sulcular epithelial cells. The initiation of the degradation of the periodontium starts with release of products from the bacteria. The bacterial products, including lipopolysaccharides (LPS), have been shown to be able to stimulate resident cells, including sulcular epithelial cells and gingival fibroblasts, but also recruited polymorphonuclear leukocytes and macrophages (Birkedal-Hansen 1993; Ding *et al.* 1996; Gemmel *et al.* 1997) to secrete pro- and anti-inflammatory cytokines and proteinases (Owen *et al.* 2004; Sorsa *et al.* 2006).

Polymorphonuclear leukocytes (PMNs) are triggered by pro-inflammatory signals and are the predominant phagocytic cells in the gingival crevice and gingival crevicular fluid (Taichman & Lindhe 1989). Increased PMN numbers have been related to the severity of gingival inflammation (Taichman & Lindhe 1989). Infiltrating PMNs in inflamed gingiva and periodontal pockets are considered to be important and essential in the pathogenesis of periodontitis due to their ability to release molecular mediators involved in both local host defence and periodontal tissue injury (Taichman & Lindhe 1989). Activated PMNs migrate through blood vessels and move directionally through tissues to bind to and phagocytose harmful microorganisms. During phagocytosis PMN subgranular and lysosomal enzymes are released not only into the phagocytic vacuole, but also extracellularly (Weiss 1989; Page 1991). The PMN enzymes that can be released into the extracellular fluid and mediate extracellular damage include several proteases with wide destructive capacity, such as serine proteinases (elastase and cathepsin G), and the matrix metalloproteinases MMP-8 (PMN collagenase) and MMP-9 (PMN gelatinase). It is noteworthy that certain MMPs such as MMP-8 and -9 have recently been found to exert, in addition to their surrogate tissue destructive properties, also unexpected protective and defensive anti-inflammatory properties (Balbin *et al.* 2003; Uitto *et al.* 2003; Sorsa *et al.* 2004; Owen *et al.* 2004; Folgueras *et al.* 2004; Gueders *et al.* 2005).

2.5 INFLAMMATION

The inflammatory response is stimulated by the release and activation of several mediators. Inflammation has been classified into acute and chronic, using the duration of the process as the criterion. The acute inflammatory process is characterised by three main stages (Scott *et al.* 1994):

1. Vasodilatation and increased blood flow to the area.
2. Increased vascular permeability with leakage of plasma from the microcirculation.
3. Migration of phagocytic leukocytes from the microcirculation into the surrounding tissue.

Among the critical processes in inflammation is the delivery of leukocytes to the site of irritation. As for example gingival or pulpal blood flow increases at the site of inflammation (due to vasodilatation and increased vascular permeability), leukocytes move to a more peripheral position in the vessels, a condition called margination. Eventually, the leukocytes roll along the endothelial wall and finally adhere to the endothelial lining. Then they move through the gaps between the endothelial cells and transmigrate along the chemotactic gradient through the basement membrane toward the site of inflammation. Bacterial products, such as LPS, acting as targets for the host's endogenous inflammatory mediators (complement components and chemokines such as IL-8) may act as chemoattractants for the consequent leukocyte recruitment and extravasation (Fouad 2002).

In the earliest stage of inflammation (the initial lesion), increased vascular leakage and the accumulation of polymorphonuclear leukocytes results in tissue oedema and the destruction of perivascular collagen fibres. The earliest stages of inflammation are probably mediated in part by the release of histamine from mast cells (Listgarten 1987). Collagenolysis has been observed due to MMP-8, MMP-9 and serine proteases (elastase, cathepsin G and proteinase 3) being released from the PMNs. However, intracellular collagen degradation by fibroblasts cannot be discounted (Listgarten 1987; Uitto *et al.* 2003; Sorsa *et al.* 2006).

2.6 NEUROGENIC INFLAMMATION

Neurogenic inflammation is a general term used to describe the local neurogenic process induced by the release of neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) from the peripheral nerve terminals of the afferent neurons. The release of these neuropeptides induces a local vasodilatation and can also be the initiating factor in the inflammatory response (**Figure 1**). This process appears to play an important role in the pathogenesis of some diseases, such as migraine (Moskowitz 1992). Moskowitz (1990) has provided a series of experiments to suggest that the pain of migraine may be a form of sterile neurogenic inflammation. Neurogenic plasma extravasation can be seen during electrical stimulation of the trigeminal ganglion in the rat (Markowitz *et al.* 1987). May and Goadsby (1999) took the view that certain disorders should be collectively regarded as neurovascular headaches to emphasise the interaction between nerves and vessels.

The vasodilatation evoked around the site of a noxious skin stimulus was suggested by Bruce (1913) to be the result of an axon reflex. The hypothesis was supported by Lewis (1937), who postulated that the flare component was mediated by local axon reflexes, called axon-reflex-mediated neurogenic inflammation. Later studies have identified the nerves involved in the vascular axon reflex to be capsaicin-sensitive, nociceptive C afferent fibres (Jancsó, Jancsó-Gábor & Szolcsányi 1967; Kenins 1981; Chahl 1988). Lewis suspected a histamine-like substance to be the transmitter substance, but today substance P is generally thought to be the primary mediator.

Activation of capsaicin-sensitive sensory nerves by antidromic electrical stimulation or by chemical irritants such as capsaicin evokes the co-release of substance P and CGRP (Lundberg *et al.* 1985). The release of these peptides from peripheral terminals of sensory neurones is then followed by increased microvascular permeability, i.e. neurogenic inflammation (Holzer 1988). Presumably, a similar mechanism may also be involved in the aetiology of several inflammatory reactions of the oral mucosa through activating the primary sensory fibres in the case of uneven tooth-crown edges and fillings standing out and irritating the mucosa as well as strong chemical stimuli and various bacterial toxins (Fazekas *et al.* 1990). Antidromic nerve stimulation or cutaneous application of irritants not only produces vasodilatation but also increases vascular permeability, thereby allowing the extravasation of plasma proteins (Jancsó *et al.* 1967, 1968) and leukocytes (Helme *et al.* 1985).
The phagocytic activity of both macrophages (Bar-Shavit *et al.* 1980; Hartung *et al.* 1986) and polymorphonuclear leukocytes (Payan *et al.* 1984) is enhanced by SP. The area of vasodilatation corresponds to the size of the receptive fields of afferent polymodal C-fibres.

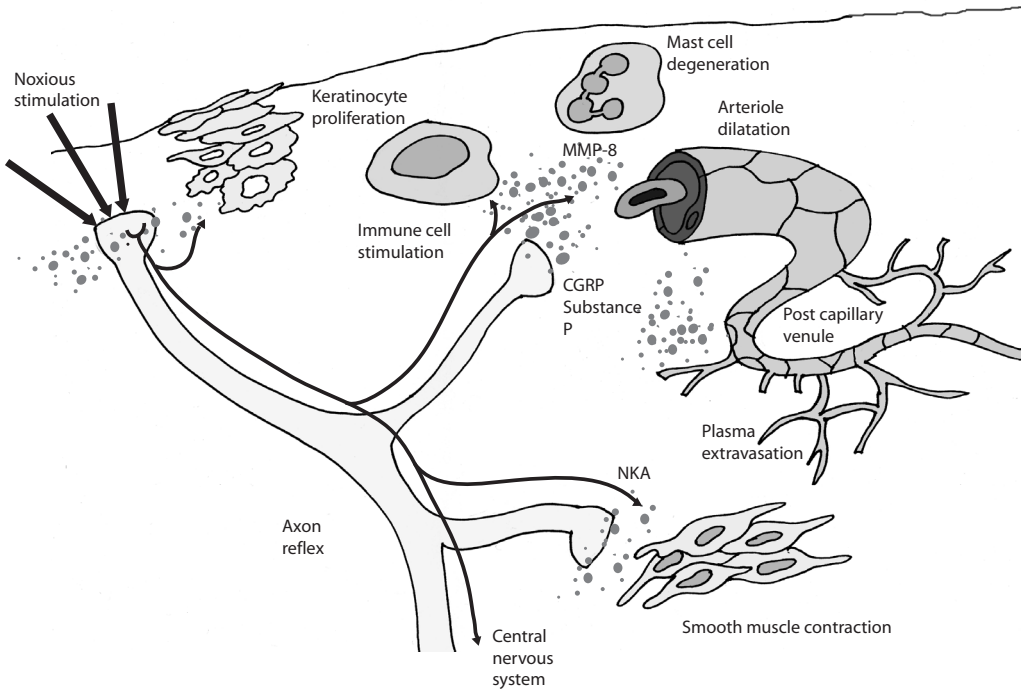

Figure 1. A schematic drawing of efferent activations of nociceptors: Pain – Inflammation – Blood flow alterations: a model of neurogenic inflammation. Upon activating C-fibres in the oral mucosa, mediators, namely neuropeptides, are released from the peripheral terminals of afferent nerves, causing characteristic symptoms of neurogenic inflammation. Also activation of cutaneous nociceptive afferents with capsaicin and painful electrical stimulation causes release of neuropeptides locally from the peripheral nerve terminals. A noxious stimulus leads to action potentials in nociceptive fibres that propagate not only to the central nervous system but also antidromically into peripheral branches. These antidromic action potentials lead to the release of MMPs and neuropeptides such as substance P (SP), calcitonin gene-related peptide (CGRP), and neurokinin A (NKA). These substances can stimulate epidermal cells and immune cells or lead to vasodilatation, plasma extravasation, and smooth muscle contraction (modified from the original figure by Ian Suk, Textbook of Pain, 2006).

2.6.1 Mediators of inflammation

Most aspects of acute inflammation are due to the action of host-derived substances rather than the direct action of the offending agent. There is also interaction between mediators. The combination of mediators present is probably more important than the presence of any one mediator in determining the physiologic response to inflammation.

2.6.1.1 Cytokines

These are a group of more than 100 proteins or peptides that modulate and control body defence and repair, including inflammation. They are described as local hormones and cell-to-cell messengers. Although most of the cytokines present in an inflammatory process are produced by inflammatory cells such as monocytes/macrophages, lymphocytes, and neutrophils, they may also be produced by a number of noninflammatory cells, which for example in the dental pulp would include fibroblasts, and endothelial cells (Fouad 2002).

Cytokines include chemotactic cytokines (chemokines), interleukins, interferons and tumour necrosis factors. Cytokines can stimulate (chemokines and pro-inflammatory cytokines) or inhibit (anti-inflammatory cytokines, interferons) the inflammation (Scott *et al.* 1994; Julkunen *et al.* 2003).

- **IL-1** has several systemic effects such as fever and the synthesis of acute-phase proteins and prostaglandins (Fouad 2002). IL-1 is a cytokine with pro-inflammatory effects. IL-1 is expressed in two isoforms: IL-1α and IL-1β. At the moment the more important immune parameter in periodontal research is interleukin 1β (IL-1β). This substance has become a focus of interest since it is known to be the most potent osteoclast-activating factor in the human organism (Dewhirst *et al.* 1985; Alexander & Damoulis 1994). IL-1β enhances bone resorption and inhibits bone formation (Nguyen *et al.* 1991). For example, certain MMPs including MMP-8 can be secreted by gingival fibroblasts stimulated by IL-1β (Abe *et al.* 2001, Cox *et al.* 2006). Stress might affect periodontal health by increasing local IL-1β levels, especially when oral hygiene is neglected (Deinzer *et al.* 1999).

- **IL-8** is a activating factor for neutrophils.

- **IL-6**, a mediator of acute inflammation, which is produced by resident cells, especially in the macrophages, in the inflamed area (Meri 2003). IL-6 was initially thought to be pro-inflammatory, but is now recognised to be an immunoregulatory and anti-inflammatory cytokine. The anti-inflammatory functions of IL-6 are caused by suppression of IL-1 and TNF-α (Tilg *et al.* 1997). IL-6 causes the formation of acute-phase proteins such as fibrinogen and C-reactive protein (CRP) in the liver.

- **TNF-α** can stimulate MMP synthesis and osteoclastic bone resorption. TNF-α may contribute to tissue degradation in periodontitis (Hanemaaijer *et al.* 1997; Tervahartiala *et al.* 2001).

2.6.1.2 Other mediators of inflammation

• **PGE_2**, prostaglandin E_2 participates in the development of the inflammatory reaction principally by potentiating the effects of other mediators rather by having a direct effect. The cyclo-oxygenase (COX) pathway leads to the generation of prostaglandins. It may be blocked by the addition of indomethacin, a prostaglandin synthetase inhibitor. Prostaglandins are also involved in the pathogenesis of pain. The direct involvement of prostanoids in for example pulpal pain has been proposed when the intravenous administration of nonsteroidal anti-inflammatory drugs (NSAIDs), which are known to block the COX pathway, resulted in significant inhibition of stimulated nerve activity in cat pulp (Ahlberg 1978; Fouad 2002).

• **Histamine** is a potent vasodilatator and mediator of vascular permeability. Histamine is found in connective tissue mast cells, basophils and platelets that are often located near blood vessels. Histamine is present in cell granules and is released by cell degranulation in response to a variety of stimuli (Fouad 2002).

• **Serotonin** (**5-hydroxytryptamine**, or **5-HT**) is a neurotransmitter synthesised in the central nervous system (CNS) and gastrointestinal tract. In the central nervous system, serotonin is believed to play an important role in the regulation of body temperature, mood, sleep, vomiting, sexuality, appetite and inflammation. Low levels of serotonin have been associated with several disorders, namely clinical depression, tension-type headache, and fibromyalgia (Bendtsen *et al.* 1997; Wolfe *et al.* 1997).

• **Bradykinin** is a powerful algesic agent and is also considered a major mediator of pain in inflammatory conditions. Specific bradykinin receptors are found on nociceptive primary afferents (Steranka *et al.* 1988), and administration of bradykinin not only generates sensory impulses but may also cause the release of transmitters from peripheral sensory neurons. Evidence to support this comes from the observation that bradykinin-induced plasma protein extravasation in the skin is reduced in animals pre-treated with capsaicin (Jancsó *et al.* 1980), a neurotoxin which selectively destroys unmyelinated sensory fibres in the skin (Jancsó *et al.* 1967).

2.7 MATRIX METALLOPROTEINASES (MMPs)

MMPs are a family of zinc-depended, structurally related but genetically distinct enzymes that degrade extracellular matrix (ECM) and basement membrane (BM) components. This group of 23 human enzymes is mainly classified based on substrate specificity and molecular structure. Humans have 24 matrixin genes including duplicated MMP-23 genes; thus there are 23 MMPs in humans (Nagase *et al*. 2006; Sorsa *et al*. 2006). MMPs are involved in physiological processes such as tissue development, remodelling and wound healing (Uitto *et al*. 2003; Sorsa *et al*. 2006), and play important roles in the regulation of cellular communication and immune functions by processing bioactive molecules including cytokines, hormones and growth factors (Sorsa *et al.* 2004). MMPs are consequently functionally active and catalytically competent at physiological pH and temperature (Nyberg *et al.* 2006). The importance of calcium ions for the optimal catalytic competence of MMPs is reflected by the inhibition of the enzyme activity by metal chelators such as EDTA and the tetracyclines (Golub *et al.* 1994; Visse & Nagase 2003).

2.7.1 General structure of MMPs

The MMPs share a common domain structure. The three common domains are the prodomain, the catalytic domain and the haemopexin-like C-terminal domain, which is linked to the catalytic domain by a flexible hinge region (Visse & Nagase 2003). The prodomain usually consists of about 80 amino acids and contains a conserved cysteine residue that can bind to the zinc atom in the catalytic domain. The disruption of this bond, usually mediated as a result of cleavage(s) of the prodomain, is required for activation of the latent proenzyme to its catalytically competent form. The C-terminal domain is thought to be involved in protein-protein interactions, determines substrate specificity and is the site for TIMP interactions.

2.7.2 Classification of the MMPs

The MMPs can be subdivided in different ways. The most commonly used groupings are based on their structural homology and substrate specificity. These six groups are 1) collagenases (MMP-1, -8 and -13), 2) gelatinases (MMP-2 and -9), 3) stromelysins (MMP-3, -10 and -11), 4) matrilysins (MMP-7 and -26), 5) membrane-type (MT-)MMPs (transmembrane type and GPI-anchored elastase) and 6) other MMPs including metalloelastase (MMP-12), enamelysin (MMP-20), epilysin (MMP-28), CA-MMP (MMP-23), and MMP-19, -21, and -27 (Visse & Nagase 2003). MMP-4, MMP-5, MMP-6 and MMP-22 are missing from the list since they were shown to be identical to other members (Nagase *et al.* 2006). It is becoming increasingly clear that these divisions are somewhat artificial as there are a number of MMPs that do not fit into any of the traditional groups.

2.7.2.1 Members of the MMP family with a role in periodontal tissue remodelling

Collagenases are capable of initiating degradation of native fibrillar collagen types I, II, III and IX (Weiss 1989). Fibroblast-type collagenase **MMP-1** is regarded to be more associated with normal tissue remodelling than with tissue degrading processes (Birkedal-Hansen 1993; Golub *et al.* 1998). **MMP-8** is synthesised by differentiating granulocytes in the bone marrow and stored in specific granules of circulating neutrophils. However, there are also other cellular sources of **MMP-8**. Expression of **MMP-13** has been reported in the pocket epithelium in human periodontitis, helping the pocket epithelium to invade into periodontal connective tissue (Tervahartiala *et al.* 2000; Kiili *et al.* 2002).

Gelatinases MMP-2 and **MMP-9** degrade gelatins (collagens that have been denatured by interstitial collagenases), collagens IV, V, VII, X and XI, elastin, and BM components (Owen & Campbell 1999).

Other MMPs also have a role in periodontal tissue remodelling. **The stromelysin** subfamily members **MMP-3** (stromelysin-1), **MMP-10** (stromelysin-2), **MMP-11** (stromelysin-3) and macrophage metalloelastase (**MMP-12**) do not themselves cleave collagen, but they act synergistically with collagenases and gelatinases. The matrilysin subgroup member **MMP-7** (**matrilysin-1**) is synthesised by epithelial cells and it can degrade several ECM and BM components as well as activate TNF- α and several proMMPs including proMMP-8 (Balbin *et al.* 1998). Membrane-type matrix metalloproteinases (MT-MMPs) mainly exert their activity on the cell surface. **MMP-14** can be detected in periodontitis-affected GCF (Tervahartiala *et al.* 2000).

2.7.3 Activation

MMPs are expressed at low levels in normal tissues, but are upregulated during inflammation (Birkedal-Hansen 1995). MMPs are mostly produced in latent, non-active form, and activation through a so-called cysteine switch is required for the enzyme function. In most cases, activation involves removal of the prodomain, resulting in lower molecular weight active forms (Nagase 1997), although the most recent studies indicate that *in vivo*, the proforms of at least certain MMPs may also be active while in full size or in complex with certain proteins (Bannikov *et al.* 2002; Fedarko *et al.* 2004). So, as MMPs are secreted as proenzymes they have to be cleaved in order to be activated. MMPs can be activated by serine proteases (trypsin and plasmin) (Sorsa *et al.* 1997; Moilanen *et al.* 2003), other MMPs, microbial proteases (Sorsa *et al.* 1992) as well as other factors such as oxygen-derived free radicals (Saari *et al.* 1990)(Figure 2). Most of the activation data have been gained from in vitro experiments, and the activation processes in vivo are not well characterised. Most likely MMP activation *in vivo* involves tissue and plasma proteinases and bacterial proteinases together with oxidative stress (Nagase 1997). Secreted MMPs are usually activated extracellularly or at the cell surface, the best-known example of cell surface activation being the activation of MMP-2 in the MMP-2/TIMP-2/MT1-MMP complex. Several MMPs may also be activated intracellularly by furin or related proprotein convertases (Nagase 1997).

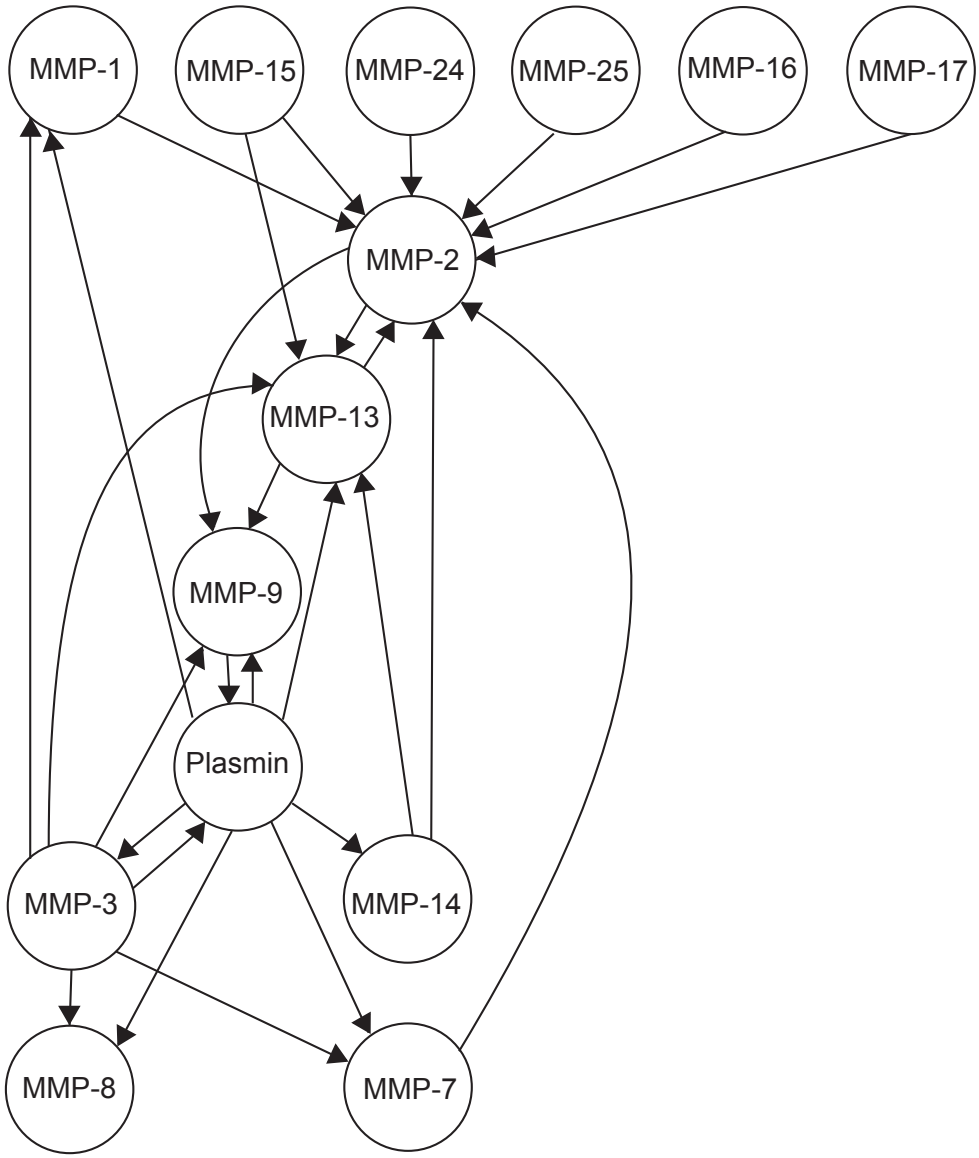

Figure 2. Activation of MMPs (source derived from: Chakraborti *et al.* 2003; Sorsa *et al.* 2006).

2.7.4 Inhibitors

MMP activity is controlled by changes in the delicate balance between the expression and synthesis of MMPs and their major endogenous inhibitors, **tissue inhibitors of matrix metalloproteinases** (**TIMPs**). Also serum α-2 macroglobulin inhibits MMPs, but TIMPs are the major group of MMP inhibitors in tissues and body fluids (Sternlicht & Werb 2001). The catalytic competence of MMPs is controlled through the activation of proenzymes, and the inhibition of the activation by TIMPs (Uitto *et al.* 2003).

TIMPs comprise a family of four protease inhibitors: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. Overall, all MMPs are inhibited by TIMPs once they are activated, but the gelatinases (MMP-2 and MMP-9) can form complexes with TIMPs when the enzymes are in the latent form. In addition to TIMPs, MMP activity can be controlled by synthetic inhibitors, which generally contain a chelating group such as EDTA, which binds tightly the catalytic zinc atom at the MMP active site. MMPs can also be inhibited by chlorhexidine (Gendron *et al.* 1999).

2.7.5 Synthetic inhibitors

Synthetic inhibition of MMPs offers an interesting possibility to control MMP-related diseases in which extensive tissue destruction is involved (Owerall & Lopez-Otin 2002). A large number of synthetic MMP inhibitors have been designed and synthesised, and some have been clinically tested for the treatment of patients with cancer or arthritis, but they have shown little efficacy so far (Baker *et al.* 2002). A number of rationally designed MMP inhibitors, such as the gelatinase-specific CTTHWGFTLC-peptide (CTT) (Koivunen *et al.* 1999; Pirilä *et al.* 2001; Heikkilä *et al.* 2006) and doxycycline and chemically modified tetracyclines (CMTs) developed by Golub *et al.* in 1987 have shown some promise in the treatment of pathologies involving excessive MMPs. Moreover, biphosphonates have been demonstrated to inhibit the activities of MMPs (Teronen *et al.* 1997, 1999; Heikkilä *et al.* 2002).

One approach in MMP inhibition is aimed at chelation of the enzyme's active site, Zn^{2+} ion (Hidalgo & Eckhardt 2001; Coussens & Fingleton 2002). The first MMP inhibitors to enter clinical trials in tumour treatment, batimastat and marimastat, base their MMP inhibitory effect on chelation (Coussens & Fingleton 2002). Tetracyclines and their non-antimicrobial analogues, chemically modified tetracyclines (CMTs), inhibit MMPs through several mechanisms. In addition to Zn^{2+} chelation, they can downregulate MMP mRNA expression, interfere with the protein processing during activation, and render the MMPs more susceptible for degradation (Golub *et al.* 1998).

2.7.6 MMP-8 (Collagenase-2) in periodontal tissue remodelling

Matrix metalloproteinase-8 (MMP-8, collagenase-2) along with MMP-1 (fibroblast-type collagenase-1) and MMP-13 (collagenase-3) are the major members of the interstitial collagenase subgroup of the MMP family. Interstitial collagenases mediate the initial step in interstitial collagen degradation by cleaving the three polypeptide chains at a single locus three-fourths of the distance of the collagen molecule from its N-terminal end (Gross & Nagai 1965). The three-fourth and one-fourth fragments generated denature spontaneously, and the denatured collagen fragments (gelatins) are susceptible to further cleavage by gelatinases (MMP-2 and MMP-9) and to lesser extent by other MMPs (including MMP-8) and gelatinolytic serine proteinases (Owen & Campbell 1999). The known ECM substrates of MMP-8 are collagens I-III, VII, X, gelatine, proteoglycans, bradykinin, substance P and pro- and anti-inflammatory cytokines/mediators (Sternlicht & Werb 2001).

MMP-8 is stored as a latent enzyme (pro-MMP-8) within the specific granules of PMN (Murphy *et al.* 1977). Pro-MMP-8 is rapidly released from activated PMN undergoing degranulation (Hasty *et al.* 1986; Balbin *et al.* 1998), and is then activated via the cysteine switch mechanism and is transformed into the active form of the enzyme (Springman *et al.* 1990). Activation can be achieved *in vitro* by organomercurials (Blaser *et al.* 1991), serine proteinases (Moilanen *et al.* 2003), MMP-3 (Knauper *et al.* 1993), microbial proteases (Sorsa *et al*. 1992) and reactive oxygen species (Saari *et al.* 1990).

Previously it was thought that the expression and release of MMP-8 was limited to neutrophils (Weiss 1989), but at present it is clear that many non-PMN-lineage cell types present in the normal and diseased human periodontium (gingival sulcular epithelial cells, fibroblasts and endothelial cells, monocyte/macrophages, plasma cells and gingival mast cells) can be induced to express distinct MMPs including MMP-8 (Hanemaaijer *et al.* 1997; Tervahartiala *et al.* 2000; Wahlgren *et al.* 2001; Kiili *et al.* 2002; Prikk *et al.* 2002; Næsse *et al.* 2003; Sorsa *et al.* 2004; Sorsa *et al.* 2006).

The expression of the 50 kD mesenchymal MMP-8 isoform has been detected in various non-PMN lineage cells, such as synovial fibroblasts and endothelial cells (Hanemaaijer *et al.* 1997), gingival sulcular epithelial cells (Tervahartiala *et al.* 2000), oral cancer cells (Moilanen *et al.* 2002, 2003) and plasma cells (Wahlgren *et al.* 2001). PMN-type MMP-8 is secreted in a latent 75-80 kD form and converted to a 65 kD active form upon PMN degranulation (Ding *et al.* 1996, 1997), and non-PMN-type 55 kD latent MMP-8 isoform is converted to a 45 kD active species upon activation (Moilanen *et al.* 2002, 2003). MMP-8 can also be found elevated and activated in high-molecular-weight bands (>100 kD) representing complexed enzyme. The high molecular-weight immunoreactivity is probably MMP-8 complexed to its endogenous inhibitors, i.e., α-2 macroglobulin (α -2M) and tissue inhibitors of matrix metalloproteinase (TIMPs), or a result of dimerisation (Ingman *et al.* 1996; Chen *et al.* 1998). The low-molecular-weight at <30 kD species most likely represents degraded fragments of MMP-8 (Apajalahti *et al.* 2003).

The major collagenase species detected in inflamed human periodontium is MMP-8 (Sorsa *et al.* 1988, 1999; Mancini *et al.* 1999; Romanelli *et al.* 1999). In contrast to a healthy patient's gingiva, extracts of untreated gingival tissue and gingival crevice fluid (GCF) from periodontitis patients contain pathologically elevated levels of collagenase-2 (MMP-8) in catalytically active form (Kiili *et al.* 2002; Sorsa *et al.* 2004, 2006). Association between increased GCF collagenase activity and progressive loss of connective tissue attachment has been demonstrated (Lee *et al.* 1995). As in other inflamed tissues, MMPs are also present in inflamed dental pulp tissue (Wahlgren *et al.* 2002) and periapical lesions (Wahlgren *et al.* 2001, 2002). MMP-8 activity and release are regulated by cytokines (tumour necrosis factor-α, interleukin-1β and interleukin-8) and various periopathogenic bacteria and their virulence factors (Ding *et al.* 1996, 1997; Hanemaaijer *et al.* 1997). On the other hand, MMP-8

seems to have an anti-tumour activity in breast carcinomas (Agarwal *et al.* 2003). Recent data from Balbin *et al.* (2003) demonstrate that MMP-8 has an unexpected role in vivo in protecting male mice from the development of skin tumours in a chemical carcinogenesis model.

MMP-8 has been considered as the prime target for anticollagenolytic adjunctive low-dose doxycycline medication in the treatment of periodontitis (Golub *et al.* 1988; Suomalainen *et al.* 1992; Sorsa *et al.* 2006; Reinhardt *et al.* 2007).

2.8 NEUROPEPTIDES

2.8.1 Function of neuropeptides

Studies using anatomical, histochemical and immunological methods have revealed the presence of a variety of peptides in afferent neurons, and based on functional evidence, it would appear that at least some of these substances play a mediator or transmitter role (Holzer 1988).

Neurons use many different chemical signals to communicate information, including neurotransmitters and peptides. Neurotransmitters, such as glutamate and aspartate, generally affect the excitability of other neurons, by depolarising or hyperpolarising them (Dickenson 1989). Peptides have much more diverse effects; among transmission, they are able to cause both vasodilatation and protein extravasation due to an increased vascular permeability (Lembeck & Holzer 1979; Gamse *et al.*1980).

Neuropeptides are released from the peripheral terminals of afferent nerve fibres. Unmyelinated and finely myelinated axons with unmyelinated terminals, which are not associated with specialised receptive structures, are termed free nerve endings. It is these unmyelinated C-fibres that are associated with the release of neuropeptides into the surrounding tissue (Scott *et al.* 1994).

Neuropeptides may contribute to the inflammatory process via several mechanisms. Firstly caused by the local vasodilatation/extravasation and followed by the release of inflammatory mediators such as histamine, PGE_2, collagenases, IL-6, and tumour necrosis factor (TNF). There is also potentiation of chemotaxis, phagocytosis and lymphocyte proliferation (Payan 1989; Hargreaves *et al.* 1994; Brain 1997).

2.8.2 Substance P

Neuropeptide **substance P (SP)** is a short-chain polypeptide that functions as a neurotransmitter and neuromodulator. It belongs to the tachykinin neuropeptide family. The endogenous receptor for SP is neurokinin 1 receptor (NK1-receptor, NK1R).

Data in the literature have shown it likely that the 11 amino acid residue polypeptide SP (Euler & Gaddum 1931) has a crucial role in the pathogenesis of neurogenic inflammation. Lembeck (1953) was the first to propose that this peptide, isolated from the intestine, may be a primary neurotransmitter at the afferent fibre terminals as well (Euler & Gaddum 1931).

In addition to SP, also other neuropeptides released from the peripheral terminals of sensory fibres can act as transmitters in evoking neurogenic inflammation (Andersson *et al.* 1988). These include CGRP, somatostatin, vasoactive intestinal peptide (VIP), neurokinin A (NKA) and neurokinin B (NKB) (Rosenfeld *et al.* 1983; Fischer *et al.* 1985; Andersson *et al.* 1988).

The release of peptides such as SP, NKA and CGRP has long been implicated in neurogenic inflammation, and many studies support the hypothesis that neuropeptides are involved in the pathophysiology of inflammatory diseases (Maggi 1995; Alstergren *et al.* 1995; Olgart 1996a). Body fluids such as saliva and gingival crevice fluid can be used in identifying markers of inflammation. The composition of these fluids changes during inflammation. In this context, the levels of the neuropeptides SP and CGRP have been measured in human saliva from patients with migraine and cluster headache (Nicoldi *et al.* 1990). Both NKA and SP evoke the release of pro-inflammatory cytokines from human monocytes (Lotz *et al.* 1988).

When an acute injury occurs, SP is released from primary afferent fibres along with other neuropeptides, such as CGRP, to cause vasodilatation of blood vessels supplying the injured area. The presence of SP also increases blood vessel permeability, facilitating the passage of cells such as macrophages and other substances essential for protective and healing processes at the injured site. SP causes the release of histamine from mast cells (Bear *et al.* 2001). The transitory nature of this response may be an important self-regulatory mechanism, as extended extravasation of inflammatory mediators to the injured area might have deleterious consequences.

Substance P can be measured with several methods including radioimmunoassay (RIA), enzyme immunoassay (EIA or ELISA) and immunohistochemistry. RIA for substance P has been developed by several groups (Powell *et al.* 1973; Yanaihara *et al.* 1976; McGregor & Bloom 1983). RIA is a quantitative method that permits measurement of tissue levels of substances rather than the anatomic location information provided by immunohistochemistry. However, in terms of safety, sensitivity and ease of handling, there have been disappointments with RIA methods. In 1982, Stjernschantz *et al.* reported an enzyme immunoassay (EIA) for substance P using a polystyrene plate coated with SP-poly-D-glutamic acid conjugate as a solid-phase antigen. The enzyme immunoassay (EIA) has been developed further by academic (Takeyama *et al.* 1990) and industrial research teams over the past decade, and nowadays the EIA method has been found to be safe and effective. On that account, we decided to concentrate on using a competitive EIA kit for substance P in this study.

2.8.3 Neuropeptides in GCF in periodontal health and disease

Many studies support the hypothesis that neuropeptides in general and tachykinins (SP and NKA) in particular are involved in the pathophysiology of inflammatory disease (Maggi 1995). Changes in the levels of SP, NKA and CGRP have previously been reported in GCF in relation to periodontal health and disease (Linden *et al.* 1997; Lundy *et al.* 1999, 2000). Bartold *et al.* (1994) have shown that SP can influence human gingival fibroblast proliferative and synthetic activity, and suggested that the action of this peptide could switch from a catabolic pro-inflammatory mode to an anabolic tissue regenerative mode depending on the presence of other factors.

Studies on innervation of the gingiva obtained from periodontitis-affected sites have provided some useful information with regard to the potential role of a neurogenic contribution to periodontal inflammation. In particular, the role of locally released neurogenic peptides as inflammagens cannot be discounted. Substance P, which is released from primary sensory afferent nerves, has significant pro-inflammatory actions (Lembech & Holzer 1979) and has been proposed to play a role in neurogenic inflammation of the periodontal tissues (Bartold *et al.* 1994). Many neurogenic peptides have been identified in inflamed gingival tissues and observed to localise throughout the connective tissues and around blood vessels (Luthman *et al.* 1989). Although these neurogenic peptides are present in healthy tissues, an upregulation of these potent bioactive molecules could have a significant impact on the initiation and establishment of the inflammatory response.

In a previous study, Linden *et al.* (1997) reported an increase in SP and NKA levels in periodontitis compared with healthy GCF, with SP having a stronger correlation with periodontitis than NKA. CGRP was not detectable at any periodontitis site, and it was concluded that some components of GCF were responsible for degrading CGRP in periodontitis (Lundy *et al.* 1999). In their later study Lundy *et al.* (2000) reported that CGRP, but not SP or NKA, is degraded by a carboxypeptidase present in GCF from periodontitis-affected sites.

The pathophysiology of periodontal disease is complex and the tachykinin SP in GCF may only play a part in the process, as it is unlikely that any single factor is responsible for the initiation and progression of disease. Therefore, it was crucial for us to study also the relationship of GCF SP levels to collagenase-2 (MMP-8), which is regarded to be among the major destructive proteases, especially in the periodontitis-affected gingival crevice fluid (GCF). Nevertheless, MMP-8 in GCF may also reflect initial protective or defensive processes (Owen *et al.* 2004, Gueders *et al.* 2005; Sorsa *et al.* 2006)

2.8.4 Neuropeptides in GCF of painful teeth

Whenever an insult causes activation of the intradental axons, the initial reaction in the pulp tissue is neurogenic vasodilatation mediated by the terminals of the afferent nerve fibres. Antidromic transmission along the collateral terminal branches of the axons results in the release of neuropeptides, which induce vasodilatation and an increase of permeability of the blood vessel walls.

Three neuropeptides are abundant in the dental pulp: SP, neurokinin A (NKA), CGRP (Akai & Wakisaka 1990). The mean levels of SP and NKA are significantly higher in gingival crevice fluid from painful teeth compared with non-painful teeth (Awawdeh *et al.* 2002b). During inflammation, sprouting of pulpal nerve fibres has been shown to be associated with increased expression of SP or CGRP closely surrounding the areas of inflammation or abscess (Kimberly *et al.* 1988; Byers 1992).

The extensive branching of the pulpal afferents also allows spreading of the neurogenic effects in a wider area of the pulp than was originally stimulated. It is also possible that activation of axons innervating the pulp and the surrounding structures may result in a spread of the neurogenic inflammatory reactions between the adjacent tissues in relatively early stages of inflammation (Olgart 1996b).

It is not clear what happens to the neuropeptides released from peptidergic nerve terminals in pulp tissue. They could be degraded locally or diffuse into the periodontal ligament through the apical foramen and, subsequently, the gingival crevice. However, after release the neuropeptides are metabolised by peptidases, which leads to termination of their activity (Lundy *et al.* 2000).

There is evidence that SP is responsible for the initiation of vasodilatation, whilst CGRP mediates the late and more dominant phase of vasodilatation in inflammation (Olgart 1996b), and there has been no evidence of a clear association between the levels of CGRP in GCF and dental pain (Awawdeh *et al.* 2002b). The aim of our present study was to compare the levels of SP and MMP-8, a potential proinflammatory compound, in human gingival crevice fluid of painfully stimulated and non-stimulated teeth.

2.9 PAINFUL STIMULATION, VAS AND LDI-IMAGING

2.9.1 Tooth pulp stimulation

There has been a recent research focus on the neurobiology of the tooth pulp, largely because of the clinical relevance of the pulp to orofacial pain and the concept that the pulp is a most useful model for studying pain by virtue of its being a pure source of nociceptive input to the CNS. When stimuli are applied through the hard tissues, only electrical stimulation (Virtanen 1991) and cold stimulation (Jyväsjärvi & Kniffki 1986) are accurate enough for reproducible activation and characterisation of pulpal nerve fibres.

The dental pulp is well known to be densely innervated by the axons of sensory nerves (Byers 1984; Fried *et al.* 1988), the activation of which can produce severe pain (Van Buren & Kleinknecht 1979). Electrical stimulation of the tooth has been used as a method for selective activation of pain pathways in both human and animal studies (Mahan & Anderson 1970; Andersson *et al.* 1973; Janhunen & Närhi 1977; Martin & Chapman 1979; Toda *et al.*1980).

In the study of Närhi *et al.*(1982), tooth stimulation currents of up to 200 μA were used, but activation of periodontal fibres was not obtained, even when applied to the periodontium. This indicates that an electrical current below 200 μA does not activate extrapulpal fibres in monopolar tooth stimulation. In the present work, the average tooth stimulation was 47 μA. Accordingly, it is most likely that only intrapulpal afferent fibres were activated during tooth stimulation in this study.

In research, painful tooth stimulation is usually performed by a constant current tooth stimulator (Närhi *et al.* 1982; Kemppainen *et al.* 1985). The electrical tooth stimulator constructed for this purpose uses mostly a monopolar electrode, where the current passes from the cathode on the intact surface of the tooth crown through the pulp to the anode, which is attached to the skin. The electrical current flows through all the tissues between the cathode and anode. The cathode of the stimulator has to be fixed to the tooth. If the electrode moves along the tooth surface there is also a change in the resistance between the electrodes and in the spreading of the current into the tooth, which affects the number of the nerve fibres activated. Monopolar tooth stimulation is a reliable method for the activation of intradental nerves both in experimental animals and human subjects. The risk of exciting nerve fibres in tissues surrounding the tooth is minimal, provided that the stimulated tooth is adequately insulated and kept dry (Virtanen 1991). By using a constant current tooth stimulator, the intensity, duration and frequency of the pulse can be precisely controlled. The optimal pulse duration is the time needed to activate a nerve fibre with minimal current intensity. The duration of the current pulse most commonly used in tooth stimulation is 10 ms and the frequency 5 Hz (Närhi 1985; Virtanen 1985).

There are two types of afferent nerve fibres within the pulp, which are activated in different ways (Närhi *et al.* 1996). Myelinated A-fibres, stimulated by cold, heat or drilling, cause fast, well-localised pain, whereas unmyelinated C-fibres, activated by stimuli which cause damage to pulp tissue, produce dull, poorly localised pain (Närhi *et al.* 1992). Electrical tooth stimulation (pulse duration 10 ms, frequency 5 Hz) at detection level activates selectively A-delta fibres, and by increasing the stimulus current it is possible to activate also pulpal C-fibres (Närhi 1985; Virtanen 1985). In addition to activation of pulpal nociceptive fibres and following pain sensation, noxious stimulation of the tooth pulp also triggers the autonomic nervous system, leading to a more widely spread systemic reaction such as changes in systemic blood pressure and heart rate (Dellow & Morgan 1969; Kemppainen *et al.* 1994, 2000a, b).

2.9.2 Capsaicin stimulation

Capsaicin, 8-methyl-N-vanillyl-6-nonenamide, is the strong ingredient in hot chili peppers of *the Capsicum* genus, and it selectively activates the unmyelinated C-fibre class of nociceptors (Caterina *et al.* 1997). Capsaicin has been shown to be a non-invasive method to activate C-nociceptive fibres in human skin (Kilo *et al.* 1995). Initial application of capsaicin to skin produces irritation and increase in sensitivity. This reaction is thought to be due to capsaicin-induced release of substance P from peripheral sensory C-fibres. After the initial exposure, capsaicin produces a long-lasting desensitisation to burn and pain (Bernstein 1988). Systemic capsaicin pre-treatment of adult rats causes a dose-dependent loss of unmyelinated fibres (Jancsó *et al.* 1987). Capsaicin is used as a therapy for temporary relief of neuralgia, and has been studied because of its properties as a stimulant of sensory nerves and neuropeptide release *in vivo* and *in vitro*. The *in vivo* effects of capsaicin on cutaneous sensation are known to differ depending on the dose and the duration of application (Green & Flammer 1988).

Activation of capsaicin-sensitive sensory nerves by electrical stimulation or by chemical irritants such as capsaicin evokes the co-release of substance P and CGRP (Lundberg *et al.* 1985). The release of these peptides from peripheral terminals of sensory neurons is followed by increased microvascular permeability, i.e. neurogenic inflammation (Holzer 1988). More recent animal studies (Fazekas *et al.* 1990; Kondo *et al.* 1995) have shown that the application of capsaicin on gingivomucosal tissues induces similar inflammatory reactions in these tissues.

2.9.3 Visual Analogue Scale (VAS)

In the present study, the subjects evaluated their pain experience to painful stimulation by the VAS method. Earlier studies have found the system to be very appropriate (Huskisson 1974; Seymour *et al.* 1985; Oliver and Knapman 1985; Wilson *et al.* 1989; Ngan *et al.* 1994; Awawdeh *et al.* 2002a, b). On the VAS scale, 0 = no pain and 100 = the worst imaginable pain intensity.

2.9.4 Laser Doppler perfusion Imaging (LDI)

A laser Doppler perfusion imager (LDI) was introduced by Nilsson, Jakobsson & Wårdell in 1990. In the LDI technique, a low-power He-Ne laser beam (wavelength 632 nm) is directed through a computer-controlled optical scanner at the tissues, which are scanned by moving the laser beam step by step in a rectangular pattern over the selected regions. With this technique, the median sampling depth is about 0.2 mm. In the presence of moving blood cells, a fraction of the light is Doppler-shifted, detected and converted into an electrical signal for further processing. The output signal is then sampled and stored by a personal computer. From the captured perfusion values a colour-coded image is generated and presented on a monitor (Wårdell *et al.* 1993; Kemppainen *et al.* 2001a, b). LDI provides relative information about the movement of red blood cells in the superficial tissue structures and the ability to instantaneously measure relative blood flow without touching the tissue.

The LDI system employs the same working principle as other conventional laser Doppler flowmeters (LDF). LDF flowmeter allows the measurement of erythrocyte flux in approximately one cubic mm of the capillary bed. However, a conventional LDF can only provide measurements of tissue perfusion at a single location, whereas the LDI system, depending on the model, allows averaging and spatial mapping of blood flow changes for a selected tissue area from 1 x 1 mm up to 50 x 50 cm in width. Thus, the LDI system is far more suitable for studying e.g. spatial and temporal characteristics of pain- and inflammation-evoked blood flow changes in the skin and gingivomucosal tissues.

The information of vascular changes in experimental and clinical inflammation sites studied with the LDI system for other tissues of the body (Wårdell *et al.* 1993) reveals its excellent value for monitoring vascular changes and clinical status in the gingiva and alveolar mucosa.

3 AIMS OF THE STUDY

The main purpose of this work was to clarify neuronally driven reflex mechanisms underlying pain-induced circulatory and inflammatory changes in human gingival tissues. Experiments were carried out especially to find out the functional role of unmyelinated nociceptive C-afferent fibres in relation to pain experience and inflammatory reactions and blood flow regulation in the gingiva.

Earlier animal studies have suggested that peripheral axons of the trigeminal nerves may cross the midline and innervate bilaterally maxillary incisors (Anderson *et al.* 1977). Here, we designed a series of experiments to determine whether experimental tooth pain or capsaicin-evoked vasodilatation in the gingiva crosses the midline of the maxilla. If this axon reflex vasodilatation crossed the midline, the experiments might show functional evidence for transmedian innervation in maxillary gingiva in humans.

Inflammatory reactions typically associated with pain have been shown to provoke expressions of pro-inflammatory mediators such as interleukin-1β, tumour necrosis factor-α and prostaglandins (Uitto *et al.* 2003; Sorsa *et al.* 2006). Matrix metalloproteinase MMP-8 is known to be the major MMP especially in periodontitis-affected GCF (Kiili *et al.* 2002). With this background, we also wished to study the effects of experimentally evoked tooth and gingival pain on GCF of MMP-8. Elevated levels of neuropeptide SP have been reported in GCF in relation to periodontal inflammation (Linden *et al.* 1997). Therefore, experiments were also performed to determine the effect of inflammatory tooth pain on GCF levels of SP.

The specific aims of this study were:

1. To clarify the spatial extensions and differences between tooth stimulation and capsaicin-induced neurogenic vasodilatation in human gingiva (*I*).

2. To study whether axon reflex vasodilatation in maxillary gingiva crosses the midline (*I*).

3. To demonstrate painful tooth and gingival stimulation evoked responses in MMP-8 levels in GCF (*II, III*).

4. To investigate whether painful tooth stimulation provokes changes in GCF SP and MMP-8 levels (*IV*).

5. To clarify the characteristics of SP and MMP-8 in tooth stimulation analysed from GCF (*IV*).

4 MATERIALS AND METHODS

Detailed methods are described in the original publications *I- IV*.

4.1 SUBJECTS, STIMULATION TECHNIQUES, MEASUREMENTS AND SAMPLES

In all *studies I, II, III* and *IV*, the subjects were healthy graduate students or researchers ranging from 21 to 45 years in age. All subjects fulfilled the following criteria: 1) no history of systemic diseases, 2) no history of antibiotics within the preceding 6 months.

Approvals for the studies were provided by the Ethics Committee of the Medical Faculty of the University of Erlangen-Nürnberg (*study I*) and the Ethics Committee of the Medical Faculty of the University of Helsinki (*studies II, III* and *IV*). All volunteers gave informed consent for participation.

In studies *I,II,III* and *IV*, during the experiments, heart rate (HR) and blood pressure (BP) responses were recorded semi-automatically from the arm by a non-invasive cuff method. These heart rate and blood pressure measurements were performed in order to clarify whether capsaicin-evoked pain and/or pulpal pain may have induced cardiovascular responses. The subjective pain levels were evaluated by a visual analogue scale, VAS (0 = no pain, 100 = the worst imaginable pain intensity).

In *study I* seven healthy human volunteers were tested in three separate experiments. All subjects were adults and had excellent oral health. For experiments one and two, filter paper moistened with capsaicin was positioned unilaterally to either the attached gingiva or the alveolar mucosa between the permanent upper first and second incisor. For the third experiment, the dental stimulation was generated with a constant-current tooth stimulator. In all three experiments, stimulation-induced blood flow changes in buccal attached gingiva and oral mucosa were mapped by laser Doppler perfusion imager (LDI; MoorLDI-MS2, Moor Instruments Ltd., UK).

Study II was performed on eight generally and periodontally healthy human adult volunteers. The dental stimulation was generated with a constant-current tooth stimulator. The stimulation period lasted 90 s. GCF samples were collected from the stimulated upper right central incisor, from the non-stimulated upper left lateral incisor, and from the non-stimulated lower right central incisor.

Samples were taken before the stimulation began and during stimulation, followed by samples taken 4 and 8 min after the end of stimulation. After one week, in the second control session GCF samples were collected similarly without tooth stimulation from six subjects. The molecular forms of MMP-8 on GCF from the stimulation and control sides were analysed by the Western immunoblot method and the MMP-8 concentrations in the GCF samples were determined by time-resolved immunofluorometric assay (IFMA).

Study III was completed with ten generally and periodontally healthy human adult volunteers. For stimulation, filter paper moistened with capsaicin was positioned in each subject unilaterally on alveolar mucosa at the site of the upper central incisor. The GCF samples were collected bilaterally from gingival crevices of several incisors in the upper jaw. The samples were taken before, during and after capsaicin stimulation. In the second control session, GCF samples were collected similarly without capsaicin stimulation from 10 subjects who had participated in the first session. The GCF samples from the stimulation and control sides were studied by Western immunoblotting. MMP-8 concentrations in the GCF samples were determined by time-resolved immunofluorometric assay (IFMA).

Six generally and periodontally healthy human adult volunteers participated in *study IV*. The dental stimulation was generated with a constant-current tooth stimulator. The stimulation period lasted 90 s. GCF samples were collected from the stimulated upper right central incisor and the non-stimulated lower right central incisor. Samples were taken before the stimulation began and during stimulation, followed by samples taken 4 and 8 min after the end of stimulation. After one week, in the second control session GCF samples were collected similarly without tooth stimulation from six subjects. The substance P concentrations in the GCF samples were determined by enzyme immuno assay (EIA) and the MMP-8 concentrations were determined by time-resolved immunofluorometric assay (IFMA).

In *studies I, II* and *IV* the cathode of the stimulator was glued to an intact upper incisor through a metal cylinder glued to the tooth. The distance from the gingival margin was at least five millimetres. The anode was attached to the arm of the subject. The cathode was surrounded by material with high resistance to prevent connection between the cathode and extrapulpal tissues. Electrode paste was applied to the cylinder to ensure contact between cathode and the tooth. The stimulator had a built-in circuit for measuring the electrode resistance. The resistances of the stimulated teeth were monitored throughout the experiment in monopolar coupling, and they ranged from 1.5 to 4 MΩ. Dental pain thresholds were always determined prior to the experiments, and they ranged from 10-18 μA. The permanent upper central incisor was electrically stimulated at an intensity of 1.5 x (*study II*) and 3 x (*study II* and *IV*) the individual pain threshold.

4.1.1 Blood flow measurements

In *study I* simulation-induced blood flow changes in buccal attached gingiva and oral mucosa were mapped by laser Doppler perfusion imager (LDI). Scanning times of 60 s (capsaicin experiments) and 90 s (tooth stimulation experiments) were used, during which the spatially mapped bilateral images from the oral mucosa and attached gingiva of the anterior maxilla could be documented. The LDI information was given on the spatial distribution of possible vasoactive changes from separate tissues simultaneously. Sympathetically maintained vasoconstriction of peripheral skin vessels was monitored by blood flow measurements on the skin of the right index finger by laser Doppler flowmetry (LDF; Periflux Pf2B, Perimed AB, Stockholm, Sweden).

4.1.2 GCF sampling

In *studies II, III* and *IV* prior to GCF sampling the sampling site was isolated with cotton rolls, and supragingival plaque was carefully removed. The region was dried with a gentle air stream, and GCF was collected by using standardised filter paper strips. The strip was placed into the crevice until mild resistance was felt, and left there for 30 s. After that, each strip was immediately placed in a polypropylene tube. The GCF volume was measured by weighing the polypropylene tube with the filter paper strip inside, before and immediately after the sample collection. Thereafter, each strip was eluted into buffer solution.

4.2 MMP-8 IMMUNOFLUOROMETRIC ASSAY (IFMA)

In *studies II, III* and *IV*, MMP-8 concentrations in the GCF samples were determined by a time-resolved immunofluorometric assay (IFMA) as described by Hanemaaijer *et al.* (1997) and Mäntylä *et al.* (2003). The monoclonal antibodies 8708 and 8706 (Medix Biochemical, Kauniainen, Finland) for MMP-8 were used as catching antibody and tracer antibody, respectively. The tracer antibody was labelled using europium-chelate (Hemmilä *et al.* 1984). The assay buffer contained 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 5 mM CaCl2, 50 µM ZnCl2, 0.5% bovine serum albumin, 0.05% sodium azide, and 20 mg/l DTPA. Samples were diluted in assay buffer and incubated for one hour, followed by incubation for one hour with tracer antibody. Enhancement solution was added, and after 5 min fluorescence was measured using 1234 Delfia Research Fluorometer (Wallac, Turku, Finland).

The monoclonal MMP-8 antibody specificity corresponded to that of the polyclonal MMP-8 antibody (Hanemaaijer *et al.* 1997; Sorsa *et al.* 1999; Mäntylä *et al.* 2003). MMP-8 concentrations (µg/l) determined during painful tooth stimulation were compared with the MMP-8 concentrations in gingivitis and periodontitis GCF published previously by Mäntylä *et al.* (2003).

4.3 MMP-8 WESTERN IMMUNOBLOTTING

In *studies II* and *III*, GCF samples from the stimulation and control sides were studied by using the ECL-Western blotting analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Samples were treated with 4 x Laemmli buffer, without reductant, and heated for 5 min at 100˚C. SDS-PAGE standards (Bio-Rad, Richmond, CA, USA) served as low range molecular weight markers. Human polymorphonuclear leukocytes (PMNs) and rheumatoid synovial fibroblast culture media, as described by Hanemaaijer *et al.* (1997), served as positive controls for PMN- and fibroblast-type MMP-8 isoforms. The samples were separated on 4 to 11% SDS-PAGE gels and then electrophoretically transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA, USA). The non-specific binding sites on the membranes were blocked with 5% dry milk powder (Valio, Finland) mixed with TTBS for one hour at 37˚C, after which the membranes were washed with TTBS six times, for 10 min each. The membranes were incubated overnight with *I* antibody, IgG fractions of specific rabbit polyclonal anti-human MMP-8 antibodies (1:250 dilution in TTBS). After six 10 min washes with TTBS, the membranes were incubated with *II* antibody, IgG fractions of ECL anti-rabbit horseradish peroxidase (1:800 dilution in TTBS) for one hour. After that, six washings with TTBS were done, each for 10 min. The molecular forms of MMP-8 on GCF were visualised by incubating the nitrocellulose membranes in ECL detection reagents, and exposing the membranes to x-ray films. The intensities of different molecular weight forms of MMP-8 were analysed with quantitated computer image scanning (Model GS-700; Bio-Rad) as described by Hanemaaijer *et al.* (1997); Kiili *et al.* (2002); Prikk *et al.* (2002); Apajalahti *et al.* (2003).

4.4 SUBSTANCE P ENZYME IMMUNOASSAY (EIA)

4.4.1 Assay procedure

We measured substance P in GCF from the stimulated and control teeth using a competitive enzyme immunoassay kit (Takeyama *et al.* 1990; Fujishima *et al.* 1997; Hanioka *et al.* 2000; Yamada *et al.* 2002) as recommended by the manufacturer. The EIA kit for SP was the Substance P immunoassay (R&D Systems Inc, Minneapolis, MN, USA). The GCF samples were diluted in EIA assay buffer to perform assays for SP. The serial diluted standards for SP were 9.8-10,000 pg/ml, and all standards and samples were assayed in duplicate. Total activity (TA), non-specific binding (NSB), maximum binding (B0), and substrate blank wells were run with each assay as means of quality control for each assay. Substance P assay is based on the competitive binding technique, in which substance P present in a sample competes with a fixed amount of alkaline phosphatase-labelled substance P for sites on a rabbit polyclonal antibody. During the incubation, the rabbit polyclonal antibody became bound

to the goat anti-rabbit antibody coated onto the 96-well microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution added to the wells determined the bound enzyme activity. Immediately following colour development, the absorbance was read at 405 nm on a microplate reader (Labsystems Multiscan RC). The intensity of the colour was inversely proportional to the concentration of substance P (SP) in the sample.

4.4.2 Calculation of results

The dublicate readings were averaged for each standard and sample and the average NSB optical density was subtracted. Serial dilutions of standards were run for each plate and their optical densities (ODs) were used in constructing a standard curve by placing the mean absorbance for each standard on a linear y-axis against the concentration on a logarithmic x-axis. The concentration of substance P was calculated from the standard curve. The highest and lowest standard of these wells was taken as a cut-off point for detectability and any reading below or over that was considered undetectable. The concentration of substance P was multiplied corresponding to the dilution. Results were expressed as ng/l (=pg/ml) in the sample.

4.5 STATISTICAL ANALYSES

In *study I*, all data (except rating) were normalised to baseline due to large inter-individual variability. The mean of the three baseline values was taken as 100%, and all succeeding values were expressed as a percent change of the individual baseline. For statistical analysis, the continuous data record was similarly reduced to average response values of three-minute time windows: one prior (= baseline) and four after the stimulation began. To compare the responses in gingival blood flow, we performed an analysis of variance (ANOVA), repeated-measure design, with the factors stimulus type (capsaicin in oral mucosa, capsaicin in attached gingiva, and tooth stimulation) and time period. Post hoc planned comparisons were performed on significant factors. Changes to baseline were tested by means of a Wilcoxon matched-pair test of the data from all subjects.

In *study II*, MMP-8 values were normalised due to large inter-individual variability. The grand mean of all 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 normalised with this grand mean (percent change as compared with grand mean). Friedman non-parametric ANOVA was then used to compare baseline, stimulation, and post-stimulation periods. In case of significance, Wilcoxon's matched-pair test was performed for *post hoc* comparisons.

In *study III* at each stimulation site, the two precapsaicin values (pre 1 and pre 2) of each subject and stimulation site were averaged to a single baseline value for ongoing analysis. Statistical comparisons between baseline and experimental positions were performed by Wilcoxon matched pair tests. Normalising was done by calculating relative changes as compared to baseline and using these values to compare responses between sites by Wilcoxon matched-pair test.

In *study IV* A Friedman non-parametric ANOVA was used to compare baseline, stimulation and post-stimulation periods for MMP-8 values and SP values. In case of significance, a Wilcoxon matched pair test was performed for *post hoc* comparisons of baseline with stimulation and with post-stimulation periods. To compare corresponding SP values of upper and lower tooth these values were normalised. For this, the grand mean of all SP values from each measurement site was calculated and each value was expressed as percent of grand mean. For comparisons, Wilcoxon's matched pair tests using these normalised values were performed. The same type of normalisation was performed on the MMP-8 values and used for the mean values shown in the figures.

In all *studies I, II, III* and *IV*, a probability value (P) of less than 0.05 was considered to represent a significant difference.

5 RESULTS

5.1 GCF FLOW, HR AND MAP

Evaluation of clinical parameters of sites exposed to tooth or capsaicin stimulation and control sites demonstrated that in addition to the general health of the volunteers, their gingival and periodontal health was excellent. GCF flow rate was not affected by painful tooth stimulation and capsaicin application. In *studies I, II, III* and *IV*, stimulation had no marked effects on systemic heart rate or mean arterial blood pressure values.

5.2 VASODILATATION RESPONSES IN TOOTH AND CAPSAICIN STIMULATION

In *study I* the average pain magnitude estimates were significantly higher during tooth stimulation than during capsaicin stimulation of the alveolar mucosa and attached gingiva. The respective maximum VAS scores were 78 ± 4, 32 ± 3, and 17 ± 4. The capsaicin-induced vasodilatation rapidly attenuated in the midline. In ipsilateral gingiva, the vasodilatation responses were affected by the stimulation paradigm: The highest responses were caused by capsaicin on alveolar mucosa, the second highest by capsaicin on attached gingiva, and the lowest effects by tooth stimulation. On contralateral gingiva, the vasodilatation responses also depended on the stimulation paradigm: the highest vasodilatation was caused by painful tooth stimulation, the second highest by capsaicin on alveolar mucosa, and the lowest effects by capsaicin on attached gingiva. High-intensity tooth stimulation induced a transient elevation in MAP and HR concomitant with a significant blood flow reduction in the finger. Neither of the capsaicin stimuli provoked any significant changes in MAP or HR responses. In comparison with capsaicin stimuli, the more painful tooth stimulation tended to induce a more marked blood flow reduction in the finger.

5.3 MMP-8 IN GCF WITH PAINFUL TOOTH STIMULATION

In *study II* the average intensity of painful tooth stimulation was 47 ± 2.2 μA. The respective average pain magnitude estimate on VAS scores was 74.4 ± 3.8, which can be considered quite a high discomfort on a scale from 0-100. Stimulation of tooth 11 significantly raised MMP-8 levels (IFMA analysis) in adjacent GCF. These pulpal-pain-evoked elevations in MMP-8 levels occurred during stimulation

and remained elevated four and eight minutes after the end of stimulation. Simultaneously, no marked changes in GCF MMP-8 levels could be detected at tooth 22 or tooth 41. The control session without stimulation showed that the repeated measurements themselves did not modulate GCF MMP-8 levels. The elevated MMP-8 levels in the GCF of the stimulated tooth were clearly lower than those of the gingivitis and periodontitis sites in our earlier study (Mäntylä *et al.* 2003). The representative Western immunoblot for molecular forms and degree of activation of MMP-8 of the stimulated incisor showed that the samples contained bands at 60 to 80 kDa corresponding to PMN-type active and pro-enzymes, and from 45 to 55 kDa corresponding to mesenchymal-type (non-PMN) active and pro-enzymes. No marked changes appeared in blots for MMP-8 of the non-stimulated teeth.

5.4 MMP-8 IN GCF WITH CAPSAICIN STIMULATION

In *study III* the average pain magnitude estimate on VAS scores was 17 ± 4, which can be considered a very mild pain level on a scale from 0-100. Capsaicin stimulation of the alveolar mucosa was effective to induce significant elevations in MMP-8 levels obtained by IFMA measurement in the GCF of the tooth at the stimulation site (= tooth 21) and of the neighbouring tooth (= tooth 22). These elevations in MMP-8 levels already began to appear during stimulation and remained for 30 min after the end of stimulation. Responses were significantly higher at the capsaicin stimulation side than on the contralateral side.

The control session without capsaicin stimulation showed that repeated measurement itself did not modulate GCF MMP-8 levels. We found that capsaicin stimulation elevated and activated the high molecular-weight-complex and PMN-type MMP-8 species and non-PMN-type MMP-8 species at the stimulation site. Capsaicin stimulation did not induce any marked changes in Western blots for MMP-8 of the teeth on the contralateral side.

5.5 MMP-8 AND SUBSTANCE P IN GCF WITH PAINFUL TOOTH STIMULATION

In *study IV* the average intensity of painful tooth stimulation was 45 ± 6.6 μA. The respective average pain magnitude estimate on VAS scores was 67.5 ± 6.3. Stimulation of the tooth 11 significantly increased the SP and MMP-8 levels in the adjacent GCF. For SP, the maximal effect was found during the stimulation period, and for the MMP-8 the most significant elevation was noted 4 min after the end of stimulation. Simultaneously, no marked changes in GCF SP or MMP-8 levels could be detected at tooth 41. Control session without stimulation showed that the repeated measurements themselves did not modulate GCF SP or MMP-8 levels.

6 DISCUSSION

In this study, capsaicin-induced inflammatory reactions in gingivomucosal tissues did not cross the midline in the anterior maxilla. The enhanced reaction found during stimulation of alveolar mucosa indicated that alveolar mucosa is more sensitive to chemical irritants than attached gingiva. These results indicated that capsaicin-evoked neurogenic inflammation in gingiva can trigger the expression and activation of MMP-8 in GCF of the adjacent teeth. It was also concluded that pulpal pain can induce local elevations in MMP-8 and SP levels in GCF.

There has been a lack of overview in the field of the possible relation of oral diseases and neurogenic inflammation. The present studies were designed to find out whether the observation on the mechanisms of the neurogenic inflammatory reactions described for other organs and tissues (Holzer 1988; Wårdell *et al.* 1993) are also valid in the gingiva and alveolar mucosa. We thus presumed that the activation of primary sensory fibres may play a role in the development of pathological reactions in those tissues of the mouth initiated and affected by bacterial toxins, metabolites, heat and masticatory forces. In general, occlusal interferences are a factor thought to contribute to the pathogenesis of periodontitis and may lead to poor response to periodontal treatment. The application of mechanical forces displaces the tooth and induces an inflammatory reaction by the compression of periodontal ligament (Giannopoulou *et al.* 2006). In a study by Giannopoulou *et al.* (2006), it was concluded that initial orthodontic tooth displacement by the use of separators induces pain and a rapid release of biochemical mediators. The study further revealed that the clinical findings in the expression of several pro-inflammatory mediators/neuropeptides, such as PGE_2, IL-1 and substance P, were associated with initial pain intensity, which is in accordance with our experimental findings. It is possible that neuropeptides, either directly or through the action of these pro-inflammatory mediators, can induce the secretion of matrix metalloproteinases.

Inflammation is a local, protective response to microbial invasion or injury. It must be precisely fine-tuned and regulated, because deficiencies or excesses of the inflammatory response cause morbidity and shorten the lifespan. The discovery that cholinergic neurons inhibit acute inflammation has qualitatively expanded our understanding of how the nervous system modulates immune responses. The nervous system reflexively regulates the inflammatory response in real time, just as it controls the heart rate and other vital functions (Tracey 2002).

Clinical inflammation and pain conditions are associated with blood flow alterations, and in some forms of pain syndromes, pain may be vascular in origin (Goadsby 1997). This project clarifies the functional role of nociceptive C-fibres in relation to a neurogenic spread of inflammation and blood flow changes in orofacial tissues. The knowledge these studies are providing is of clinical significance since it attempts to explain the mechanisms and the key substances such as SP and MMP-8 in the pathogenesis of neurogenic inflammatory reactions in human gingival tissues. Furthermore, the results of this project will be opening new and more adequate diagnostic and treatment possibilities of periodontal and orofacial inflammation.

Furthermore, it is becoming clear that bidirectional communication exists between sensory nerves and inflammatory/immune cells, sustained by release of chemical factors from both sides. The net result of such cross-communication is the establishment of a positive feedback cycle that could play an important role in the genesis and maintenance of both acute and chronic inflammatory processes. The importance of the interaction between the nervous system and immune system signalling has been demonstrated recently in the development of pathological pain. Watkins and Maier (2002) have proposed that cytokines produced by inflammatory and glial cells change neuronal excitability and that this link contributes directly to the development of intractable pain. The ultimate goal of future functional studies of pain and inflammatory reactions in orofacial regions, including teeth and periodontium, is to improve our understanding of how to avoid insults and how to cure inflammation.

6.1 THE SPATIAL EXTENSIONS AND DIFFERENCES BETWEEN TOOTH STIMULATION AND CAPSAICIN-INDUCED NEUROGENIC VASODILATATION IN HUMAN GINGIVA (*I*)

In *study I*, capsaicin stimuli of the gingivomucosal tissues evoked a pronounced vasodilatation in ipsilateral gingiva that rapidly attenuated at the midline of the anterior maxilla. In contrast to this, painful stimulation of the upper central incisor produced comparable blood flow elevations bilaterally in the maxillary gingiva. The contralateral vasodilatations were increased as a function of increasing stimulus-evoked pain estimates. These results indicate that axon-reflex-mediated vasodilatation in the gingiva does not cross the midline of the maxilla, and importantly, that pain may contribute to trigeminal blood flow alterations in humans.

Since axon-reflex vasodilatation is known to spread symmetrically around the nociceptive stimulus and corresponds to the size of the receptive fields of stimulated nociceptive afferents (Wårdell *et al.* 1993), the present results do not favour the hypothesis of functional transmedian innervation of gingival tissues in anterior maxilla, or that peripheral axons are crossing the midline of anterior maxilla in significant numbers. A similar asymmetric blood flow response in relation to the midline

has been found in the skin of the posterior part of the neck (Mentis & Lynn 1992). Our results are also in agreement with those from several anatomical (Fuller *et al.* 1979; Byers & Matthews 1981) and electrophysiological (Saag & Reid 1981; Foster & Robinson 1994) studies in animals showing that cross-innervation of maxillary and mandibular nerves exists rarely, if at all.

LDI, which was used in our study, provides relative information about the movement of red blood cells in the superficial tissue structures and the ability to instantaneously measure relative gingival blood flow without touching the tissue. These features and the sensitivity of the waveform to measure blood flow changes suggest its potential value for monitoring clinical status and early disease detection. In our investigation, we applied the LDI technique for the first time to record blood flow in human gingiva. The current finding – that tooth stimulation evokes bilateral vasodilatation while capsaicin stimulation of the gingiva mainly produces unilateral vasodilatation –emphasises the usefulness of LDI in clarifying spatial features of neurogenic vasoactive changes in the intra-oral tissues.

In our investigation, the contralateral vasodilatations during tooth and capsaicin stimulation were increased as a function of increasing stimulus-evoked pain responses. Interestingly, blood flow increases in the maxillary attached gingiva and oral mucosa were evoked bilaterally, not unilaterally. We hypothesised that such vasodilatations in the oral mucosa might be induced by the autonomic nervous system and mediated via parasympathetic vasodilator fibres. In other words, the current stimulation-related blood flow increase in oral mucosa and attached gingiva in the contralateral region could be based on centrally mediated, parasympathetic vasodilator mechanisms (Izumi & Karita 1992).

In the current study, painful stimuli of the tooth induced a short blood flow decrease in the skin of the finger. These blood flow decreases occurred simultaneously with the transient pain-induced HR and BP elevation. It has been shown that various stimuli causing arousal, mental stress and pain produce an increase in sympathetic nerve traffic and are associated with transient reflex vasoconstriction in the human skin (Lundberg *et al.* 1989; Wallin 1990). Thus, the most convincing explanation for the present pain-induced blood flow decrease in the skin of the finger is a sympathetic vasoconstrictor reflex.

6.2 PAINFUL TOOTH AND GINGIVAL STIMULATION-EVOKED RESPONSES IN MMP-8 LEVELS IN GCF (*II*, *III*)

6.2.1 Painful tooth stimulation

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

The average intensity of the tooth pulp stimulation was 47 μA, when the upper right central incisor (tooth 11) was electrically stimulated at an intensity of 3x the individual pain threshold. First we studied the tooth pulp stimulation at 1.5x the individual pain threshold with six volunteers (unpublished data), but as it did not have a significant effect on MMP-8 levels, in further experiments 3x the individual pain threshold was used (average current intensity 47 μA). Only the high intensity tooth stimulation induced increased elevations in MMP-8 levels. The high-intensity stimulus used (47 μA) activates not only pulpal A-delta fibres but also C afferent fibres (Närhi 1985; Virtanen 1985; Kemppainen *et al.* 2001a, b). Electrophysiological evidence indicates that the maximal strength (below 100 μA) of the electrical stimulation used in the present study for the dental stimulation, even when applied to the periodontal tissues, does not activate extrapulpal fibres (Närhi *et al.* 1982).

It is known that MMP-8 is present at very low levels in periodontally healthy subjects' GCF (Mäntylä *et al.* 2003). In our study, experimental tooth pain induced significant elevation in GCF MMP-8 levels (reaching the average levels of 45-55 μg/L) of periodontally healthy volunteers. In patients having gingivitis or periodontitis the GCF MMP-8 levels reach up to 750 μg/l or 2500 μg/l, respectively (Mäntylä *et al.* 2003). Thus the present elevated MMP-8 levels in the GCF of the painfully stimulated tooth were clearly lower than those of the gingivitis and periodontitis sites in our earlier study (Mäntylä *et al.* 2003). The physiological relevance of the present results is that experimental tooth pain triggers an active process with increased levels of proteolytic enzymes in GCF similarly to e.g. those seen with tooth pain in symptomatic pulpitis (Awawdeh *et al.* 2002a) or caused by orthodontic tooth movement (Apajalahti *et al.* 2003, Giannopoulou *et al.* 2006). More generally, the GCF of teeth with elevated levels of MMP-8 regardless of the cause (tooth pain, gingivitis, periodontitis) may be at potentially elevated risk for increased/rapid periodontal breakdown in population with occlusal overload caused e.g. by bruxism or extensive occlusal interferences. In other words, the pulpal pain does not induce an increase in GCF MMP-8 levels comparable to periodontitis-affected GCF, but it is possible that the slight increase in GCF MMP-8 levels induced by pain does not alone cause tissue destruction, although it may well participate in tissue destruction when associated with other tissue damage promoting factors.

6.2.2 Capsaicin stimulation

In *study III*, capsaicin stimulation of the alveolar mucosa evoked significant GCF MMP-8 elevations and activations of the ipsilateral incisors of the anterior maxilla. No significant increases could be detected in MMP-8 levels in GCF contralaterally to the capsaicin stimulation. These results indicate that chemical irritation of the gingiva by capsaicin can provoke a local neurogenic inflammation with enhanced proteolytic potential in the closely adjacent gingival tissues, which reaction does not cross the midline in the anterior of the maxilla. Since capsaicin provoked matrix degrading enzymes in GCF, the study shows further evidence for a functional link between neurogenic inflammation and periodontal tissue destruction in humans.

Similarly to capsaicin-induced neurogenic vasodilatation in gingiva in *study I*, the capsaicin-evoked response in GCF of MMP-8 did not cross the midline of the anterior maxilla. It is likely that the capsaicin-evoked GCF MMP-8 elevation is based on an axon-reflex-mediated neurogenic inflammatory reaction in human gingivomucosal tissues. According to these results it is also possible that, similarly to the experimentally induced neurogenic inflammation, clinical gingival inflammatory reactions elicited by bacterial toxins and metabolites do not spread from one side to the other of the anterior maxilla.

6.3 PAINFUL TOOTH STIMULATION-INDUCED RELEASE OF SP AND MMP-8 IN GCF (*IV*)

In *study IV*, 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 to this, the GCF SP and MMP-8 levels of the ipsilateral lower incisor remained unchanged. This study is the first to report that painful stimulation of a tooth provokes significant elevations in GCF in both MMP-8 and SP levels of the stimulated tooth. These results indicate that painful tooth stimulation can induce local neurogenic inflammatory responses with enhanced 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.

Animal studies have shown the existence of branched nerves innervating both intrapulpal and periodontal tissues (Foster & Robinson 1994). Branches of sensory nerves can innervate both adjacent pulps and surrounding periodontal tissues. On that account, when the branch that innervates the pulp is stimulated, the entire neurone depolarises, including branches innervating adjacent teeth and adjacent periodontal tissues. A noxious stimulus leads to rapid action potentials in nociceptive fibres that propagate not only to the central nervous system but also antidromically into peripheral

branches. According to this, the present data support the possibility of a 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 (Sorsa *et al.* 2004). Alternatively, depending on the role of MMP-8 due to its ability to modulate anti-inflammatory cytokines and chemokines, this reaction may also be, at least in part, anti-inflammatory or defensive (Owen *et al.* 2004; Gueders *et al.* 2005).

Pulpal pain and inflammation lead to an increase in intrapulpal pressure (Byers & Närhi 1999). It is possible that an increase in the rate of fluid flow out of the root canal system, carrying with it released neuropeptides and MMP-8, could explain the increased levels of SP and MMP-8 found in the GCF of stimulated teeth. There are direct communication channels between the pulp cavity and the periodontal ligament by way of apical foramina, accessory lateral and furcation canals and dentinal tubules (Seltzer 1984; Trowbridge *et al.* 1998). In our study, none of the teeth sampled had gingival recession and it is therefore unlikely that SP and MMP-8 were carried into the gingival crevice from the pulp in dentinal fluid. Markers have been shown to migrate from the pulp to all areas of the periodontal ligament, including the marginal gingiva (Walton & Langeland 1978). However, although it is possible that neuropeptides in GCF could originate from the dental pulp, the long diffusion pathway that lowers concentrations by several orders of magnitude (Ghazi *et al.* 2000) would tend to argue against this hypothesis (Awawdeh *et al.* 2002).

6.4 THE CHARACTERISTICS OF SP AND MMP-8 IN TOOTH STIMULATION ANALYSED FROM GCF (*IV*)

This cross-sectional *study IV* demonstrated the significantly elevated SP and MMP-8 levels in GCF from painful teeth compared to GCF from non-painful teeth. These findings indicate that experimental pulpal pain can produce local elevations in GCF levels of pro-inflammatory neuropeptide SP and a potent host-tissue destructive or defensive protease, MMP-8.

Clinical studies have shown that SP (Awawdeh *et al.* 2002b) and MMP-8 (Wahlgren *et al.* 2002) are more abundant in pulp tissues from painful compared to healthy human teeth. Moreover, tooth pain in symptomatic pulpitis (Awawdeh *et al.* 2002a) or caused by orthodontic tooth movement (Apajalahti *et al.* 2003; Giannopoulou *et al.* 2006) is associated with elevated GCF MMP-8 and SP levels of these teeth. These clinical findings indicate that painful teeth may be allied with elevated levels of pro/anti-inflammatory SP and MMP-8 both in pulp tissue and in adjacent GCF. Now when an association between MMP-8 and SP levels can be established, the neuropeptides and MMPs in GCF might potentially be utilised as an indirect non-invasive diagnostic method of assessing pulpal status.

There is accumulating evidence that the nervous system can modulate inflammation in inflammatory diseases (Lowman *et al.* 1988; Naukkarinen *et al.* 1993; Scott *et al.* 1994). Mast cells are commonly found in close association with peripheral nerves (Scott *et al.* 1994) that may secrete neuropeptides (SP, VIP), which can trigger degranulation of the mast cells (Bunker *et al.* 1991; Scott *et al.* 1994). The previous finding by Næsse *et al.* (2003) postulates that mast cells in the gingival infiltrate can express MMP-8. Thus it is tempting to speculate that pulpal pain firstly evoked GCF SP release from the nerve terminals, which possibly triggered the consequent GCF MMP-8 elevations from mast cells and/or other inflammatory/immune cells.

GCF can be collected by several techniques: with filter paper strips, micropipette tubes using gingival washing and capillary tubing. Before we started the data collection in *study IV*, we performed a pilot study with seventeen volunteers (unpublished data) in order to find the most adequate method of collecting both MMP-8 and substance P. In this pilot study the GCF was collected in different ways, i.e. using the microdialysis method. Microdialysis permits collection of inflammatory mediators in awake dental pain patients who can simultaneously provide verbal pain reports (Hargreaves & Costello 1990). We used micropipette tubes based on capillary tubing and Ringer's solution as a vehicle to carry MMPs and neuropeptides to the eppendorf tube. However, in terms of sensitivity, this method was less than satisfactory for us to detect both neuropeptides and MMPs. Finally we decided to concentrate on harvesting GCF by filter paper strips, which is the most commonly used method for GCF collection.

The fact that teeth which are not in occlusion have lower neuropeptide levels than teeth in occlusion (Kvinnsland & Heyeraas 1992; Byers & Närhi 1999) supports the hypothesis that neuropeptides may have a role in the pathophysiology of periodontal destruction in painful teeth with occlusal interferences and in bruxism. Moreover, these findings may also be the first step in studying these pulp-based neurogenic processes in the future in population suffering from periodontal problems simultaneously with bruxism, occlusal interferences and non-physiological strong masticatory forces.

7 CONCLUSIONS

1. Capsaicin produces an axon-reflex-mediated neurogenic inflammatory reaction in human gingivomucosal tissues that does not cross the midline of the anterior maxilla. The enhancement of this reaction during mucosal stimulation suggests that alveolar mucosa has a higher susceptibility than attached gingiva to inflammatory effects induced by chemical irritants in the oral cavity. The more extended vasoactive changes in contralateral gingivomucosal tissues during different stimuli are most likely based on a pain-evoked, possibly parasympathetic, vasoactive reflex mechanism.

2. Painful tooth stimulation can induce local inflammatory responses with enhanced proteolytic potential in the very adjacent gingival tissues. Pulpal pain can contribute to the local regulation of MMP-8 levels in GCF. Pulpal stimulation-evoked increase in the GCF MMP-8 levels of the stimulated tooth could result from a local neurogenic, possibly axon-reflex-mediated, pro- or anti-inflammatory mechanism.

3. Chemical irritation of gingivomucosal tissues by capsaicin produces local elevation of the levels of a potent host-tissue destructive protease, MMP-8, in GCF of the neighbouring teeth. Similarly to the capsaicin-induced neurogenic vasodilatation in gingiva, the capsaicin-evoked response in GCF of MMP-8 does not cross the midline of the anterior maxilla. It is likely that capsaicin-evoked GCF MMP-8 elevation is based on an axon-reflex-mediated neurogenic inflammatory reaction in human gingivomucosal tissues. It is possible that, similarly to the experimentally induced neurogenic inflammation, clinical gingival inflammatory reactions elicited by bacterial toxins and metabolites do not spread from one side of the anterior maxilla to the other.

4. Experimental pulpal pain can produce local elevations in GCF levels of MMP-8 and substance P, the transmitter in evoking neurogenic inflammation. Analysis of these data supports the possibility of a local neurogenic spread of inflammation from intrapulpal to surrounding periodontal tissues. Neurogenic inflammatory response, on the other hand, may also be important for starting tissue protecting anti-inflammatory reactions. Thus, the pulp-based neurogenic process may predispose such sites to the progression of periodontal destruction and/or represent defence against gingival inflammation.

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