MATRIX METALLOPROTEINASES (MMPS) IN THE DENTIN-PULP COMPLEX OF HEALTHY AND CARIOUS TEETH

MERJA SULKALA

Faculty of Medicine,
Institute of Dentistry,
University of Oulu

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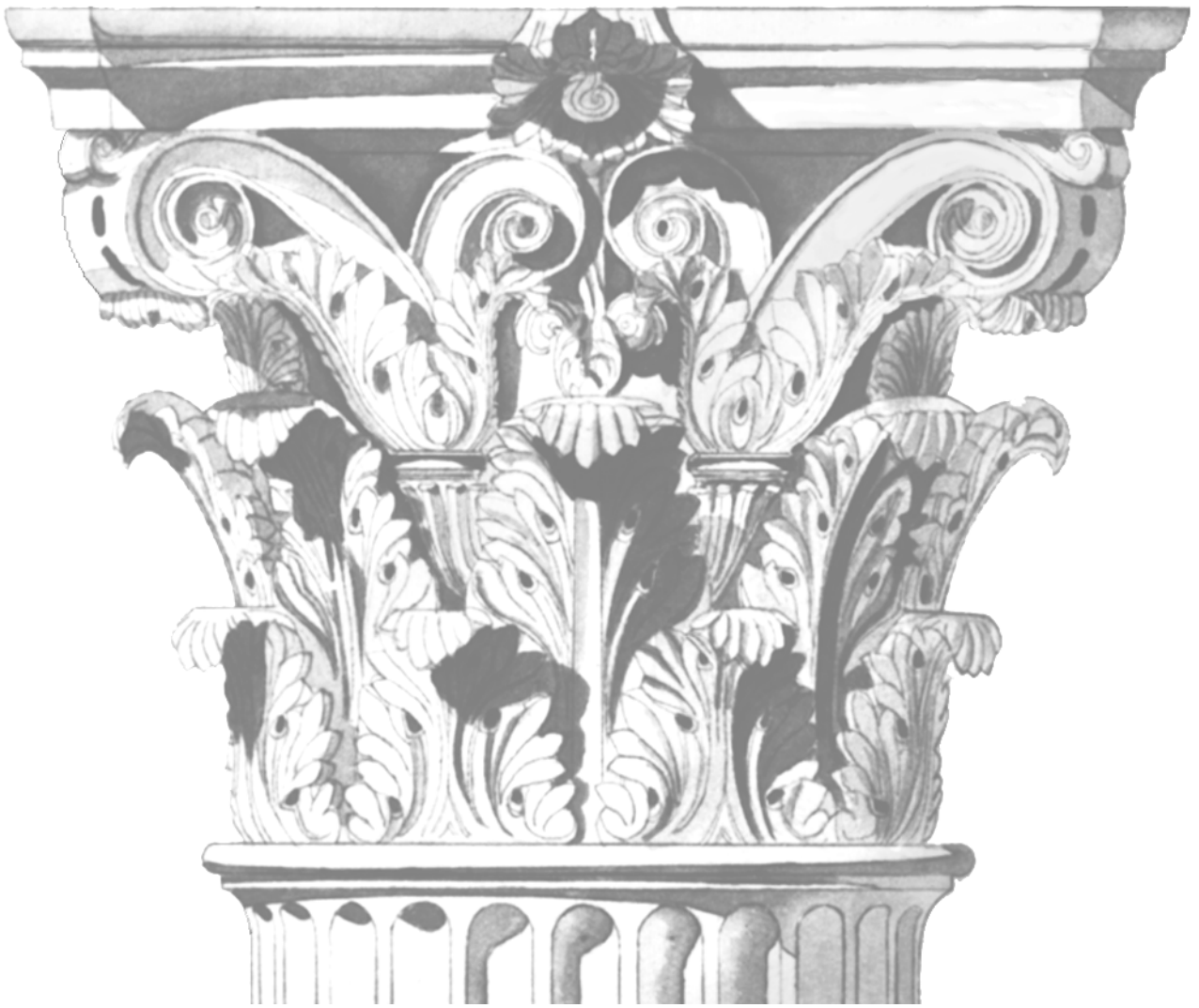

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Supervised by
Docent Leo Tjäderhane
Professor Tuula Salo
Professor Markku Larmas

Reviewed by
Docent Pirjo-Liisa Lukinmaa
Professor Veli-Jukka Uitto

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Faculty of Medicine, Institute of Dentistry, University of Oulu, P.O.Box 5281, FIN-90014 University of Oulu, Finland
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Abstract

The dentin-pulp complex comprises mineralized dentin and the vital soft tissues encased inside dentin, *i.e.* odontoblasts and pulp tissue. During caries progression, the dentinal minerals are dissolved and eventually the collagenous organic matrix is degraded. However, the exact mechanisms and enzymes responsible for the organic matrix breakdown remain unknown. Matrix metalloproteinases (MMPs), a family of endopeptidases capable of degrading in concert virtually all extracellular matrix components, are expressed during normal dentin-pulp complex formation and maintenance. MMP activity has also been suggested to contribute to the organic matrix degradation during dentin caries progression and to the repair and defense reactions elicited by caries in the dentin-pulp complex cells. The aim of the study was to further elucidate the role of host MMPs in dentin caries progression and the origin of MMPs in carious dentin as well as the possible changes in MMP expression in the cells of the dentin-pulp complex in response to caries.

MMP inhibitors decreased the area of dentin caries lesions *in vivo*, suggesting the involvement of host MMPs in dentin caries pathogenesis. When the overall MMP gene expression was examined by cDNA microarray, pooled pulp samples demonstrated a high level of MMP-13 expression, but failed to show any unequivocal changes in MMP expression due to caries. MMP-13 expression is rare among normal human adult tissues. Real-time quantitative PCR of individual pulp and odontoblast samples demonstrated a rather large variation in relative MMP-13 mRNA expression between samples, especially pulp samples. Protein expression of MMP-13 was detected in pulp and odontoblasts without any major differences between the tissues of sound and carious teeth. This was also the case with the MMP-20 (enamelysin) protein, which was demonstrated in odontoblasts and the pulp tissue of fully developed human teeth. MMP-20, MMP-8, and gelatinases (especially MMP-2) were demonstrated in human dentin, and dentinal MMPs exhibited activity against native and denatured type I collagen when released.

The study demonstrates the presence of MMPs in the soft and hard tissue compartments of the dentin-pulp complex. These enzymes may also contribute to dentin caries progression and response reactions to caries.

Keywords: dental caries, dental pulp, dentin, matrix metalloproteinase inhibitors, matrix metalloproteinases, odontoblasts

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Oulu, November 2004 Merja Sulkala

Abbreviations

AEC	3-amino-9-ethylcarbazole
ANOVA	analysis of variance
APMA	4-aminophenyl mercuric acetate
BP	bisphosphonate
BSA	bovine serum albumine
cDNA	complementary deoxy-ribonucleic acid
cpm	counts *per* minute
CMC	carboxymethyl cellulose
CMT	chemically modified tetracycline
C_T	threshold cycle
DTT	dithiothreitol
dUTP	2'-deoxyuridine 5'-triphosphate
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
DTPA	diethylenetriaminepentaacetic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCF	gingival crevicular fluid
HSD	honestly significant difference
IHC	immunohistochemistry, immunohistochemical
IFMA	immunofluorometric analysis
kDa	kilodalton
MDPF	2-methoxy- 2,4 diphenyl-3(2H) furanone
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MT	membrane type
NCBI	(United States) National Center for Biotechnology Information
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMN	polymorphonuclear

PVDF	polyvinylene difluoride
rpm	rounds *per* minute
RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulphate
SIBLING	small integrin-binding ligand N-linked glycoprotein
STD	standard deviation
TBS	Tris-buffered saline
TC	tetracycline
TCA	trichloroacetic acid
TGF-β	transforming growth factor beta
TIMP	tissue inhibitor of metalloproteinases

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Sulkala M, Wahlgren J, Larmas M, Sorsa T, Teronen O, Salo T & Tjäderhane L (2001) The effects of MMP inhibitors on human salivary MMP activity and caries progression in rats. J Dent Res 80: 1545-1549.

II Sulkala M, Pääkkönen V, Larmas M, Salo T & Tjäderhane L (2004) Matrix metalloproteinase-13 (MMP-13, collagenase-3) is highly expressed in human tooth pulp. Connect Tissue Res 45: 1-7.

III Sulkala M, Larmas M, Sorsa T, Salo T & Tjäderhane L (2002) The localization of matrix metalloproteinase-20 (MMP-20, enamelysin) in mature human teeth. J Dent Res 81: 603-607.

IV Sulkala M, Tervahartiala T, Sorsa T, Larmas M, Salo T & Tjäderhane L. Collagenases and gelatinases in human dentin. Submitted for publication.

Contents

1 Introduction

The dentin-pulp complex consists of a tubular mineralized hard tissue compartment, dentin, and the vital soft tissue encompassed by and partly embedded into the dentin, *i.e.* odontoblasts and pulp tissue. Dental caries is a multifactorial disease that requires a susceptible host as well as cariogenic bacteria and diet in order to develop (Keyes 1962). Caries results in hard tissue loss, which occurs in dentin by dissolution of dentinal minerals and, eventually, by degradation of the collagenous organic matrix (Johansen & Parks 1961, Frank *et al.* 1989, Schüpbach *et al.* 1989, Nyvad & Fejerskov 1990). Even though organic matrix breakdown is essential for lesion progression (Katz *et al.* 1987), the exact mechanisms and enzymes responsible for it are largely unknown.

The members of the matrix metalloproteinase (MMP) enzyme family are in concert capable of degrading virtually all extracellular matrix components (Kähäri & Saarialho-Kere 1999). Several MMPs are found in normal dentin-pulp complex cells and tissues, and they are considered to be involved in many physiological processes during the formation and maintenance of the dentin-pulp complex (*e.g.* Hall *et al.* 1999, Martin-De Las Heras *et al.* 2000, Palosaari *et al.* 2000, 2002, 2003). However, their activity has also been suggested to contribute to the organic matrix degradation during dentin caries progression (Dung *et al.* 1995, Tjäderhane *et al.* 1998a, 1999) and to the repair and defense reactions elicited by caries in dentin-pulp complex cells (Palosaari *et al.* 2000, Tjäderhane *et al.* 2001a).

In this study, the role of host MMPs in dentin caries progression was examined by means of an *in vivo* experiment with MMP inhibitors. The MMP gene and protein expression in dentin-pulp complex cells as well as the presence of MMPs in dentin were investigated to elucidate the source of MMPs in carious dentin and the possible changes in MMP expression and synthesis in response to caries.

2 Review of the literature

2.1 Structure and formation of the dentin-pulp complex

The external morphology of a tooth is dictated by dentin, which can be further divided anatomically into coronal dentin, covered by enamel, and radicular dentin, covered by cementum. Dental pulp, the soft tissue compartment maintaining the tooth's vitality, is encased inside dentin. The term 'dentin-pulp complex' emphasizes the structural and functional integration of dentin and dental pulp (Torneck 1994, Pashley 1996), even though these tissues differ markedly with respect to their chemical composition and cellular components (Linde & Goldberg 1993, Goldberg & Smith 2004).

2.1.1 Odontoblasts

Odontoblasts, the cells responsible for physiological dentin formation, make up a single-cell layer lining the walls of the pulp space. They originate in the neural crest and are thus termed ectomesenchymal cells. Odontoblast differentiation from the cell pool of the dental papilla involves reciprocal signaling with the adjacent cells of the dental epithelium. This signaling is mediated by transcription factors, growth factors, cell surface receptors, and matrix molecules. Having exited the cell cycle, odontoblasts are presumably no longer able to divide. (ten Cate 1994, Thesleff & Sharpe 1997, Garant 2003a.)

A differentiated odontoblast is a highly polarized cell consisting of a cell body facing towards the center of the pulp space and a cell process extending into predentin and dentin inside a dentinal tubule. The shape of the odontoblast cell body varies from columnar in the coronal area to flattened in the apical area (Torneck 1994). A typical columnar cell body is 20–40 μm tall and 3–5 μm wide. It has a proximally positioned nucleus, and its cytoplasm contains microtubules and filaments as well as organelles for protein synthesis and secretion and energy supplementation. (Linde & Goldberg 1993.)

Mitochondria, rough endoplasmic reticulum, and Golgi apparatus are abundant during rapid dentin formation, but less abundant in odontoblasts during the later stages of development (Couve 1986). Differentiated odontoblasts are connected to each other by junctional complexes (Linde & Goldberg 1993), which effectively isolate the dentin formation zone from the rest of the pulp (Turner *et al.* 1989, Bishop & Yoshida 1992).

The odontoblast process consists of a 0.5–1.0 µm thick main trunk and thinner (0.1–0.2 µm thick) lateral branches. The process contains cell organelles responsible for exo- and endocytosis, and it is rich in intermediate filaments and microtubules (Linde & Goldberg 1993). The odontoblast process extends at least halfway through the mineralized dentin, even though cellular elements presumably of odontoblastic origin have been observed throughout the full thickness of dentin (Linde & Goldberg 1993, Torneck 1994, Garant 2003b).

2.1.2 Pulp proper

Proximal to the odontoblastic layer is the pulp proper, soft tissue consisting of extracellular matrix, tissue fluid, and a cellular compartment including fibroblasts, undifferentiated mesenchymal cells, immunocompetent cells as well as vascular and neural elements. (Torneck 1994, Goldberg & Smith 2004.)

The pulp extracellular matrix (ECM) contains collagens, proteoglycans, glycoproteins, and water in a composition similar to other soft connective tissues (Linde 1985, Torneck 1994). Most of the pulp collagen is fibrillar. The type I and III collagens account for over 95% of total collagen, while the types V and VI are less abundant. (Tsuzaki *et al.* 1990, Lukinmaa & Waltimo 1992.)

Fibroblasts are the most common cells in the pulp. In the coronal pulp they are arranged into a cell-rich zone (Höhl cells, subodontoblastic cells) separated from the odontoblasts by a cell-free zone (Weil's basal layer). (Linde & Goldberg 1993, Torneck 1994.) In young teeth fibroblasts are characterized by a prominent cytoplasm with numerous cell organelles reflecting a high rate of protein synthesis and secretion. In older teeth fibroblasts have a flattened, often spindle-shaped morphology. Undifferentiated mesenchymal cells, the progenitor cells of the pulp, are scattered throughout the pulp, often located adjacent to blood vessels. These large cells have few cellular organelles, indicating a low level of metabolic activity. Upon aging the number of cells in the pulp decreases, leading to a decreased cell/matrix ratio. (Torneck 1994, Mjör *et al.* 2001.)

Most of the immunocompetent cells in the pulp are dendritic cells and macrophages, presenting antigens to other immune cells. Especially macrophages also display a marked potential for phagocytosis. Normal pulp contains various T-lymphocytes, but only a few studies have been able to demonstrate B-lymphocytes and plasma cells in normal human pulp tissue. (Jontell *et al.* 1998.)

The blood vessels and nerves enter the pulp as a neurovascular bundle through the apical foramen or foramina in the root apical area, from where they branch coronally. The vasculature is especially rich in the subodontoblastic region, from where some capillaries even enter the interodontoblastic spaces (Kramer 1960, Takahashi *et al.* 1982), but not

into dentin (Linde & Goldberg 1993). The sensory afferent and sympathetic nerves are also arranged into a rich network with terminal endings in the subodontoblastic region (comprising the plexus of Raschkow in the coronal pulp), and some of them enter dentinal tubules in the vicinity of the odontoblastic process. (Torneck 1994, Mjör *et al.* 2001.)

2.1.3 Structure and formation of dentin

Dentin can be considered a mineralized connective tissue, because the extracellular matrix largely dictates its characteristics. On an average, dentin contains 50% volume minerals, mainly hydroxyapatite, 30% organic matrix, and 20% water. However, the distribution of these constituents varies in different parts of dentin. (Linde & Goldberg 1993.)

2.1.3.1 Primary and secondary dentinogenesis

The process of dentin formation is called dentinogenesis. Primary dentin, which comprises the bulk of the tooth and is formed during the rapid phase of physiological dentin formation, is mainly a product of odontoblasts (Linde & Goldberg 1993). Dentin formation begins at the late bell stage of the tooth germ in the coronal area, from where the commencement of dentin formation proceeds into the apical direction (ten Cate 1994).

The first dentin layer, the *mantle dentin* approximately 5–30 µm thick, differs from the rest of the primary dentin with respect to its ECM components (Linde & Goldberg 1993). Initial mantle dentin mineralization is different from the other parts of dentin in that it occurs *via* matrix vesicle formation (Stratmann *et al.* 1996, 1997). When mantle dentin mineralization begins, odontoblasts push out a cell extension, which will later form the odontoblast process. This extension is left behind, and the odontoblasts form junctional complexes with each other (ten Cate 1994).

After mantle dentin formation, primary dentinogenesis proceeds as the deposition of *circumpulpal dentin*. Odontoblasts secrete an organic matrix, which is organized and then mineralized. The zone of organization, a 10–20 µm wide zone of predentin, remains non-mineralized throughout the lifetime of the tooth. It contains collagen and other ECM molecules, but due to active secretion and elimination of the constituents, its composition differs from the extracellular matrix of mineralized dentin. (Linde & Goldberg 1993.) Due to the junctional complexes between the odontoblasts, circumpulpal dentin is thought to be produced exclusively by odontoblasts (ten Cate 1994). However, some components may be of pulpal origin, as suggested by the presence of bundles of collagen fibrils extending from the pulp into predentin ("von Korff fibers"), at least during the later stages of physiological dentin formation (Bishop *et al.* 1991).

Mineralization of the circumpulpal dentin takes place at the distal border of predentin, in the mineralization zone. Initiation of mineralization and crystal growth are actively promoted and controlled by specific, often anionic, ECM proteins (Boskey 2003). The literature on the mechanisms of dentin mineralization concentrates on the role of calcium (Linde & Goldberg 1993). Calcium ions are concentrated from the tissue fluid of the pulp into predentin (Lundgren *et al.* 1992), the ions being transported through the odontoblastic intracellular pathway involving Ca-ATPase and Na^+/Ca^{2+} exchangers bound to vesicles and plasma membrane, and possibly also *via* the intercellular pathway (Linde & Goldberg 1993).

When producing dentin, odontoblasts move in a centripetal direction, leaving their processes into the mineralized dentin. As a consequence, 1–3 μm wide branched dentinal tubules are formed into the circumpulpal dentin (Linde & Goldberg 1993), being more numerous in the proximal than the distal dentin (Mjör & Nordahl 1996). In addition to odontoblast processes, dentinal tubules contain bundles of collagen fibrils, especially in the pulpal parts (Dai *et al.* 1991), some proteoglycans and glycoproteins, and possibly a hyaluronidase-susceptible layer (lamina limitans) on the walls of the tubules (Torneck 1998, Garant 2003b). The dentinal tubules also contain tissue fluid of dentin, dentinal fluid, which is normal extracellular fluid with respect to its inorganic content (Larmas *et al.* 1986). Serum proteins are also found in dentinal fluid, but in ratios different from serum (Torneck 1998.)

The dentin formed at the predentin-mineralization front is called *intertubular dentin*. At some distance distally from predentin a 0.5–1.0 μm thick collar inside the dentin tubule is formed. It is often called *peritubular dentin*, but it would be called more precisely *intratubular dentin* (Linde & Goldberg 1993, Mjör *et al.* 2001). Intratubular dentin contains less collagen and more minerals than intertubular dentin (Frank 1959, Torneck 1994, Mjör *et al.* 2001). Its formation process has been debated, but at least its formation requires a viable odontoblast (Linde & Goldberg 1993, ten Cate 1994). *Physiological dentin sclerosis*, occurring in both coronal and radicular dentin (Stanley *et al.* 1983), has been suggested to result from continuous deposition of intratubular dentin (Torneck 1994, Mjör *et al.* 2001). Over time, intratubular dentin formation may practically occlude the tubule, decreasing the permeability of dentin significantly. Therefore, it can be considered a physiological defensive reaction of the dentin-pulp complex, which protects the cells of the dentin-pulp complex from external irritation (Pashley 1996).

The time point of primary dentinogenesis turning into slower *secondary dentinogenesis* has been defined as the time of the tooth reaching occlusal contact (Linde & Goldberg 1993), the time of the closure of the apex (ten Cate 1994, Garant 2003b) or the time of tooth eruption (Mjör *et al.* 2001). Nevertheless, by definition, secondary dentin is deposited at a slower rate throughout the pulp space by the original primary odontoblasts. Even though it is basically similar to primary dentin, it may be less mineralized than primary dentin (ten Cate 1994, Garant 2003b), and its tubularity may be less regular compared to primary dentin (Torneck 1994).

2.1.3.2 Tertiary dentinogenesis

The term *tertiary dentin* has been used to describe the dentin formed due to an external non-physiological stimulus to the tooth. It is formed only underneath the site of injury, and its deposition rate is proportional to the degree of injury. (Linde & Goldberg 1993, ten Cate 1994, Mjör *et al*. 2001.) Based on the type of dentin-forming cells, tertiary dentin can also be called *reactionary dentin*, when synthesized by primary odontoblasts, or *reparative dentin*, when formed by the replacement odontoblasts with usually a more fibroblast-like phenotype (Lesot *et al*. 1993, Smith *et al.* 1995). Indeed, pulp contains stem cells, which are capable of self-renewal and differentiation into a variety of different cells, and which regenerate a pulp-like soft tissue with tubular dentin-like mineralization (Gronthos *et al.* 2000, 2002).

The structure of tertiary dentin varies greatly, being often less regular and less mineralized than primary dentin (Mjör *et al.* 2001). It may be separated from physiological dentin by *interface dentin* (Mjör *et al.* 2001) or a *calciotraumatic line* (Linde & Goldberg 1993), which is thought to protect the cells of the pulp by closing the tubular connection between the pulp and the affected physiological dentin. Occasionally, in severe injuries involving a high rate of tertiary dentin formation, some odontoblasts may become trapped into the atubular tertiary dentin, forming *osteodentin* (Torneck 1994).

2.1.4 Dentin extracellular matrix

The dentin extracellular framework, into and on which the minerals are embedded, is also called *organic matrix*, with reference to its role as a template for mineralization and its presumable regulative activity on mineralization. It is composed of collagenous and non-collagenous proteins, proteoglycans, and lipids, its composition varying depending on the species and location of dentin. (Linde & Goldberg 1993.)

Collagens comprise over 90% of the dentin organic matrix, type I collagen being the most abundant collagen (Gage 1984, Linde & Goldberg 1993). Most of the type I collagen in dentin consists of triple helices of two α_1(I) and one α_2(I) chains, but at least rodent and bovine odontoblasts synthesize a type I trimer consisting of three identical α_1(I) chains (Munksgaard & Moe 1980, Sodek & Mandell 1982). However, its presence in human odontoblasts and dentin is unclear. In predentin, sparse small-diameter collagen fibrils are packed into thicker and densely packed bundles (Goldberg & Septier 1989, Beniash *et al.* 2000), forming a dentinal collagen matrix that is highly insoluble and resistant to swelling (Veis & Schlueter 1964).

Type III collagen is expressed and synthesized by human odontoblasts (Lukinmaa *et al.* 1993, Palosaari *et al.* 2001), and it has been observed in human intratubular dentin and predentin as well as inside the dentinal tubules (Becker *et al.* 1986, Waltimo *et al.* 1994), but not in normal intertubular dentin. However, it has been demonstrated in dentin affected by the generalized, hereditary connective tissue disease osteogenesis imperfecta, (Lukinmaa 1988) and in reparative dentin (Magloire *et al.* 1988). Type VI collagen has

been localized in human predentin and intratubular dentin (Becker *et al.* 1986), whereas type V collagen has only been found in predentin but not in dentin (Lukinmaa & Waltimo 1992).

Many non-collagenous ECM proteins are considered important in actively promoting and controlling the mineralization of collagen fibrils and crystal growth during dentin formation (Butler *et al.* 2003). Dentin proteoglycans, consisting of protein core and glycosaminoglycan side chains, are thought also to contribute to the arrangement of collagen fibrils. Some proteoglycans are secreted into proximal predentin and subsequently processed, whereas some are secreted close to the mineralization front. (Linde & Goldberg 1993.) Sialic acid-rich proteins of the SIBLING (small integrin-binding ligand N-linked glycoprotein) family are characteristic of mineralized tissues, but not uniformly distributed among them. Dentin contains large amounts of dentin sialoprotein and dentin phosphoproteins compared to bone, for example (Butler *et al.* 2003, Goldberg & Smith 2004). Dentin non-collagenous ECM also contains plasma proteins and several growth factors (Linde & Goldberg 1993).

2.2 Dental caries

Dental caries can be defined as a bacterial disease that leads to a loss of the calcified tissues of the tooth. Actually, for caries development and progression interplay of host, including saliva and teeth, microbial flora and dietary factors, with caries-favoring dynamics is required to be present for a sufficient time (Keyes 1962, Newbrun 1989). Caries also elicits various response reactions in the dental pulp (Smith 2002).

Caries lesions can be classified according to the site of caries initiation into coronal caries (pit and fissure caries, smooth surface caries), root caries, and secondary caries at the margins of dental restorations. The lesions also differ in respect to their rate of progression, *i.e.* activity, and can be categorized into rapid or slowly progressing, or even arrested lesions. Due to differences in composition, primarily in the degree of mineralization, caries progression involves different processes in different tooth tissues. Enamel caries progression can be regarded essentially as a demineralization process, since enamel contains 95% of minerals by weight (ten Cate 1994). During caries progression in cementum and dentin, the mineral phase is dissolved, but the degradation of the specialized organic matrix plays a more central role than in enamel.

Cariogenic microbes, including bacteria and yeasts, are able to produce organic acids by fermentation of carbohydrates. Microbes reside in the dental plaque on the surface and inside the caries lesion, and their metabolism, which is affected by dietary factors and oral hygiene, dictates the pH conditions of the caries lesion. (Edwarsson 1986, Björndal & Mjör 2001.) Dietary carbohydrates induce the enrichment of acid-tolerating, aciduric bacteria in the dental plaque (van Houte 1994, Kleinberg 2002).

Caries development has been suggested to be initiated and promoted by a few cariogenic bacteria (van Houte 1994), and caries could therefore be regarded as an infectious disease. Actinomyces species and especially mutans streptococci, including *Streptococcus mutans* and *Streptococcus sobrinus*, are associated with caries initiation

and progression. Lactobacilli are often predominant in advanced lesions, and they are associated with the progression of the lesion, but rarely with its initiation. (van Houte 1980, 1994, Edwarsson 1986.) On the other hand, the variability in the frequency and proportions of the bacterial groups, especially in dentin caries lesions, is notable (Edwarssson 1986, Kleinberg 2002). Therefore, the current mixed-bacterial/ecological approach to dental caries causation considers a relative increase of any acidogenic bacteria as the microbial component favoring caries development (Kleinberg 2002).

2.2.1 Progression of dentin caries

Caries progression in dentin has been suggested to involve first dentin demineralization by bacterial acids followed by bacterial invasion and organic matrix breakdown (Johansen & Parks 1961, Katz *et al.* 1987, Frank *et al.* 1989, Schüpbach *et al.* 1989, Nyvad & Fejerskov 1990, Tjäderhane *et al.* 1998a). Dentin demineralization affects both inter- and intratubular dentin (Arends *et al.* 1989, Fusayama 1991), and active caries progression is associated with a lactate-dominant acid profile and low (even below 5) pH of the lesion (Hojo *et al.* 1994). However, dentinal minerals dissolve more easily than those of enamel, even at pH 6.7 (Hoppenbrouwers *et al.* 1986, 1987).

Due to the presence of organic components of dentin, acids alone do not produce caries-like lesions in dentin *in vitro*, but the launching enzymatic proteolysis in mildly acidic conditions leads to cavity formation (Katz *et al.* 1987). The degradation of the organic matrix also promotes further caries progression by enhancing dentin demineralization (Kleter *et al.* 1994). The non-collagenous components of the dentin organic matrix are altered during caries progression even before the bacterial invasion, but the collagenous matrix is well protected when embedded in minerals (Lormée *et al.* 1986). The dentin organic matrix is susceptible to collagenases only after dentin demineralization (Evans & Prophet 1950, Klont & ten Cate 1991b). Also, since a bacterial collagenase degrades the dentin matrix in a remineralization solution but not in a demineralization solution, it has been suggested that proteolysis occurs during the remineralization phase (Kawasaki & Featherstone 1997). However, the pH optimum for the bacterial enzyme used in the study (Kawasaki & Featherstone 1997) is close to neutral, and the activity was reduced directly by the acidic pH. Nevertheless, dentin demineralization and the degradation of the organic matrix could also occur simultaneously due to the rather high critical pH of dentin (Hoppenbrouwers *et al.* 1986, 1987).

Proteolytic enzymes of host and/or microbial origin demonstrating collagenase-like activity (Mäkinen 1970) as well as exo- and endopeptidase activities (Larmas *et al.* 1968, Larmas 1972) have been detected in carious dentin, especially in the dentinal tubules. Native fibrillar collagens, including the major dentin collagen, type I, are rather resistant to proteases other than collagenases (Birkedal-Hansen & Dano 1981, Ryhänen *et al.* 1983). Vertebrate collagenases cleave the α-chains of these native fibrillar collagens into characteristic 3/4 length (αA) and 1/4 length (αB) fragments, which are rapidly denatured and become susceptible to non-specific proteases. (Dung & Liu 1999, Kähäri &

Saarialho-Kere 1999). In contrast, bacterial collagenases can cleave each polypeptide chain of collagen in the triple-helical form at more than 200 sites (Dung & Liu 1999).

Collagenolytic activity has been demonstrated in only a few oral bacteria, and mostly in species unrelated to dental caries (Dung & Liu 1999). In an early work of Burnett & Scherp (1951) high proportions of proteolytic bacteria were observed in the superficial layers of dentin caries lesions. Later *Streptococcus mutans* has been shown to produce metalloprotease gelatinases, which degrade synthetic collagen-related peptide (Jackson *et al.* 1997) and tendon and skin collagens (Rosengren & Winblad 1976). The cariogenic yeast *Candida albicans* also possesses a collagenolytic enzyme that is able to degrade dentinal collagen in acidic conditions (Kaminishi *et al.* 1986, Hagihara *et al.* 1988). However, there are also contradictory data showing that cariogenic microbial flora possess only minor activity against collagen in the native or even in a denatured form (gelatin) (Tjäderhane *et al.* 1998a, Dung & Liu 1999) or no activity against dentin collagen (van Strijp *et al.* 1997). Likewise, retention of demineralized dentin slabs in the oral cavity results in degradation of the organic matrix to a notably varying extent, but no correlation between dentin collagenolysis and the gelatinolytic or collagenolytic activity of the predominant microbial flora has been observed (van Strijp *et al.* 1994, 1997).

Bacterial acids have been suggested to modify dentinal collagen, making it more susceptible to non-specific proteases (Larmas 1986, Klont *et al.* 1991). The proportion of denatured trypsin-susceptible collagen of demineralized dentin varies notably from 1 up to 30% of total collagen, depending on the incubation conditions (Scott & Leaver 1974, Klont *et al.* 1991, van Strijp *et al.* 1992). However, true carious dentin is more resistant to proteolytic degradation by trypsin (Young & Massler 1963) and by true collagenases (Prophet & Atkinson 1953, Konetzka *et al.* 1956, Armstrong 1958) than demineralized sound dentin, suggesting marked differences between *in vitro* demineralized dentin and true carious dentin.

In the experiments by Scott and Leaver (1974) the susceptibility of dentin collagen to non-specific proteases was observed to be higher than that of tendon collagen, which was suggested to be due to an endogenous collagenase in dentin. Indeed, a latent collagenolytic enzyme that is activated by trypsin was found later (Dayan *et al.* 1983, Dumas *et al.* 1985). This dentinal collagenase may also be activated by acid or even water treatment *in vitro* (Dung *et al.* 1995). Dentin is one possible source of the host matrix metalloproteinases (MMPs) detected in soft carious dentin (MMP-2, -8, and -9) (Tjäderhane *et al.* 1998a), but they may also originate from odontoblasts (or even pulp) *via* dentinal tubules and saliva. Salivary MMPs are activated by pH changes typical of caries lesions *in vitro* (Tjäderhane *et al.* 1998a).

In the deep layers of dentin caries lesions with partial demineralization, where bacteria have not invaded (Ohgushi & Fusayama 1975), the crossbands of organic matrix collagen fibrils are recoverable, which means that they may remineralize (Kuboki *et al.* 1977). Remineralization protects the organic matrix from degradation. However, the presence of a demineralized matrix is not essential for remineralization, since remineralization may occur on residual crystals. (Klont & ten Cate 1991a.) Lesion arrest is characterized by increased resistance to acids and proteases compared to active caries lesions (Young & Massler 1963), which suggests molecular alterations both in the mineral phase and in the organic matrix with lesion arrest.

2.2.2 Response of the dentin-pulp complex to caries

The dentin-pulp complex exhibits various responses to caries, including events of injury, defense, and repair (Pashley 1996, Smith 2002). These responses are reactions in the vital tissues of the tooth in combination with physico-chemical processes involving the mineral phase of the tooth. The magnitude and nature of the response reflects the extent of the injury, but also the prevailing conditions of the dentin-pulp complex (Pashley 1996, Smith 2002).

Bacterial acids and other metabolites, even though diluted and mixed with dentinal constituents, are noxious to the cells of the dentin-pulp complex, and due to the tubular structure of dentin, they may affect the cells at a rather early phase of caries (Pashley 1996, Smith 2002). Superficial active enamel caries is enough to induce changes in the organelles, morphology, and enzymatic activity of odontoblast cells as well as a decrease in predentin width and the accumulation of cells into the cell-free zone (Larmas 1986, Magloire *et al.* 1992, Björndal *et al.* 1998). In pulp the metabolic activity of cells increases at an early phase of dentinal caries (Kobayashi *et al.* 1996).

The first sign of pulpal defense is a local inflammation under the site of irritation, which can be observed at the early stage of caries affecting only the enamel (Brännström & Lind 1965). Despite its defensive nature, inflammation may lead to further tissue injury (Smith 2002). The inflammatory cell infiltrate first consists mainly of T-lymphocytes, which are later accompanied by macrophages, neutrophils, B-lymphocytes, and plasma cells (Izumi *et al.* 1995). Dendritic cells presenting antigens gather into the area below the caries lesion in co-distribution with nerve fibers (Sakurai *et al.* 1999, Yoshiba *et al.* 2003). The inflammatory reaction in the pulp also involves a local increase in interstitial pressure (Heyeraas & Berggreen 1999) and possibly an increase in the blood flow of the non-functional capillaries in the area of injury (Mjör *et al.* 2001). An outward movement of dentinal fluid (Vongsavan & Matthews 1991, 1992), restricting the inward movement of possibly noxious substances, is promoted by the inflammatory reaction (Pashley 1996, Heyeraas & Berggreen 1999). Even though odontoblasts physiologically control the dentinal fluid flow and contents in fully developed teeth (Turner *et al.* 1989), severe injuries may disturb the junctional complexes of the odontoblastic layer enough to cause pulpal proteins (Turner *et al.* 1989) or even inflammatory cells (Bergenholtz 1977) to leak into dentinal tubules.

Repair processes aim to overcome the effects of the injury, *e.g.* to restore the distance between the dental pulp and the oral cavity, thus contributing significantly to the defense of the dentin-pulp complex (Pashley 1996, Smith 2002). Primary odontoblasts synthesize reactionary dentin during mild forms of injury, *e.g.* under precavitated enamel caries and slowly progressing dentin caries lesions (Björndal *et al.* 1998, Björndal & Darvann 1999), but deep dentinal caries lesions frequently cause the death of primary odontoblasts followed by recruitment and differentiation of replacement odontoblasts (Björndal & Darvann 1999). Repair processes may also involve angiogenesis, *i.e.* the formation of new capillaries, in the pulp (Pashley 1996, Smith 2002). The signals for tissue repair are thought to be mediated by dentin-bound growth factors released during caries progression (Magloire *et al.* 1992, Smith & Lesot 2001).

Dentin permeability, influenced by both the density and the diameter of the dentinal tubules, has a major effect on the extent of injury to the dentin-pulp complex cells by the noxious components (Pashley 1996). Dentin permeability may be decreased by tertiary dentin formation and by obliteration of the tubules, through either physiological dentin sclerosis that is evenly distributed in dentin or *reactive dentin sclerosis* observed in carious teeth beneath and next to the caries lesion (Stanley *et al.* 1983, Tagami *et al.* 1992). Reactive dentin sclerosis may result from the acceleration of intratubular dentinogenesis, but also by the precipitation of dissolved minerals (Daculsi *et al*. 1979, Fusayama 1991, Björndal *et al.* 1998, Arnold *et al.* 2001).

2.3 Matrix metalloproteinases

Matrix metalloproteinases (MMPs), matrixins, are a family of enzymes capable of degrading in concert essentially all extracellular matrix components (for reviews see Kähäri & Saarialho-Kere 1999, Sternlicht & Werb 2001, Visse & Nagase 2003). They also participate in the processing of a variety of other substrates, *e.g.* other proteinases, latent growth factors, cell surface receptors, and chemotactic molecules. MMPs contribute to various physiological processes, *e.g.* embryonic development, tissue turnover, and wound healing, as well as to pathological processes, *e.g.* cancer, arthritis, periodontitis, and fibrosis. The structural resemblance to matrix metalloproteinase-1 (the first MMP discovered), the dependence on Zn^{2+} and Ca^{2+} to function, and the inhibition by tissue inhibitors of metalloproteinases (TIMPs) are features to assign a protease to the MMP family. The MMP family contains 23 members in humans (Table 1). On the basis of substrate specificity and homology, MMPs can be divided into six groups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs (MT-MMPs), and other MMPs. (Murphy & Knäuper 1997, Sternlicht & Werb 2001, Visse & Nagase 2003.)

All MMPs except MMP-23 are expressed and translated with an amino-terminal signal peptide, which targets the protein to the rough endoplasmic reticulum and most of them to be secreted. After the signal peptide, the MMPs contain a highly conserved pro-domain, which generally maintains the enzyme in the zymogen form, and a catalytic domain responsible for substrate hydrolysis and autolytic cleavages of the MMP molecule (Murphy & Knäuper 1997, Sternlicht & Werb 2001.) The catalytic domain contains a zinc ion bound to three histidines and, in the non-activated form, to the cysteine residue of the pro-domain, and an additional structural zinc ion and 2-3 calcium ions required for enzymatic activity and stability. In all secreted MMPs except MMP-7 and -26 the catalytic domain is followed by a C-terminal hemopexin-like or vitronectin-like domain contributing to substrate and TIMP binding, proteolytic activity, and membrane activation; in most cases there is a connecting hinge region between these domains. In MT-MMPs the C-terminal domain attaches the molecule to the plasma membrane. (Sternlicht & Werb 2001, Visse & Nagase 2003.)

The biological activity of MMPs can be regulated at the level of gene transcription, compartmentalization, enzyme activation, and inactivation (Sternlicht & Werb 2001,

Visse & Nagase 2003). The transcriptional level of most MMPs is regulated by, for instance, growth factors, hormones, and cytokines. However, constitutive expression is characteristic for some MMPs, *e.g.* MMP-2 and MT1-MMP. The ultimate level of MMP synthesis is also regulated by mRNA processing. (Sternlicht & Werb 2001.)

After translation, most MMPs are readily secreted, but at least MMP-7, -8, and -9 can be stored intracellularly (Kähäri & Saarialho-Kere 1999). The spatial restriction of MMP activity, often to the pericellular regions, is achieved by the expression of membrane-bound MMPs and cell surface receptors for MMPs or MMP-activating enzymes. The distribution and expression levels of MMP inhibitors also regulate the local MMP activity. MMP activity can also be abolished by proteolytic processing, even though the loss of some domains may not always totally inactivate the enzyme. The clearance of intact enzymes can occur *via* binding to a specific receptor or by complexing with, for example, α_2-macroglobulin for endocytosis and degradation. (Sternlicht & Werb 2001.)

The substrate spectrum *in vitro* for each MMP is quite well documented (Table 1), but the data on MMP substrates *in vivo* are more restricted. Possibly due to the overlapping substrate spectrum, the lack of an MMP in knockout animals seldom results in severe complications or a lethal phenotype *per se*. (Sternlicht & Werb 2001.)

Table 1. Human matrix metalloproteinases

Group	MMP	Other names	Molecular weight[a]	Substrates *in vitro*
collagenases	MMP-1	Collagenase-1, interstitial collagenase, fibroblast collagenase	52; 43, 41	Type I, II, III, VII, VIII, X, XI collagens, gelatin, fibronectin, laminin, tenascin, α_2-macroglobulin, IL-1β, proTNF-α, proMMP-1, -2 and -9
	MMP-8	Collagenase-2, neutrophil collagenase	85; 64 (PMN) – 55-65; 50, 46, 42 (mesenchymal)	Type I, II, III collagens, aggrecan, fibrinogen, α_2-macroglobulin, bradykinin
	MMP-13	Collagenase-3	65, 55; 42	Type I, II, III, IV, VI, IX, X, XIV collagens, collagen telopeptides, gelatin, fibronectin, tenascin-C, aggrecan, fibrinogen, α_2-macroglobulin, proMMP-9
gelatinases	MMP-2	Gelatinase A, 72 kDa gelatinase / type IV collagenase	72; 67, 62	Type I, II, III, IV, V, VII, X, XI collagens, gelatin, laminin, elastin, fibronectin, α_2-macroglobulin, IL-1β, proTNF-α, latent TGF-β, proMMP-1, -2, -9, and -13
	MMP-9	Gelatinase B, 92 kDa gelatinase / type IV collagenase, type V collagenase	92; 82 (PMN) – 76; 67 (other cells)	Type IV, V, XI, XIV collagens, gelatin, elastin, laminin, aggrecan, α_2-macroglobulin, IL-1β, proTNF-α

Table 1. Continued.

Group	MMP	Other names	Molecular weight[a]	Substrates *in vitro*
stromelysins	MMP-3	Stromelysin-1, transin, proteoglycanase, collagenase activating protein (CAP)	57; 45, 28	Type III, IV, V, VII, IX, X, XI collagens, collagen telopeptides, gelatin, elastin, fibronectin, laminin, aggrecan, decorin, perlecan, versican, α_2-macroglobulin, IL-1β, proTNF-α, fibrinogen, proMMP-1, -3, -7, -8, -9, and -13
	MMP-10	Stromelysin-2, transin-2	52; 44	Type III, IV, V collagens, gelatin, elastin, fibronectin, aggrecan, proMMP-1, -7, -8 and -9
	MMP-11	Stromelysin-3	58, 51; 46, 28	Type IV collagen, gelatin, fibronectin, α_2-proteinase inhibitor
matrilysins	MMP-7	Matrilysin-1, putative metalloprotease (PUMP-1), Matrin	28; 19	Type I, IV collagens, gelatin, elastin, fibronectin, laminin, aggrecan, α_2-macroglobulin, proTNF-α, proMMP-1, -2, -7 and -9
	MMP-26	Matrilysin-2	29; 19	Gelatin, fibronectin, α_2-macroglobulin, fibrinogen, proMMP-9
MT-MMPs	MMP-14	MT1-MMP	66; 54	Type I, II, III collagens, gelatin, fibronectin, laminin, aggrecan, α_2-macroglobulin, proTNF-α, fibrinogen, proMMP-2, -13 and -20
	MMP-15	MT2-MMP	71, 68; 62	Fibronectin, tenascin, laminin, aggrecan, proTNF-α, proMMP-2
	MMP-16	MT3-MMP	64; 55	Type III collagen, gelatin, fibronectin, laminin, α_2-macroglobulin, proMMP-2
	MMP-17	MT4-MMP	71; 67	Gelatin, fibrinogen, fibrin, proTNF-α
	MMP-24	MT5-MMP	73; 64	Fibronectin, gelatin, chondroitin sulphate proteoglycan, proMMP-2
	MMP-25	MT6-MMP, Leukolysin	62; 58	Type IV collagen, gelatin, fibronectin, fibrinogen, fibrin, proMMP-2
Other MMPs	MMP-12	Macrophage elastase, metalloelastase	54; 45, 22	Type I, IV, V collagens, elastin, gelatin, fibronectin, laminin, aggrecan, α_2-macroglobulin, proTNF-α, fibrinogen
	MMP-19	Matrix metalloproteinase RASI-1	57; 45	Type IV collagen, gelatin, laminin, fibronectin, fibrinogen, fibrin

Table 1. Continued.

Group	MMP	Other names	Molecular weight[a]	Substrates *in vitro*
	MMP-20	Enamelysin	54; 43, 22	Amelogenin, type IV collagen, aggrecan, fibronectin, laminin, tenascin-C
	MMP-21	-	62; 49	α1-antitrypsin
	MMP-23 (genes A and B)	Cysteine array (CA-) MMP	44; (–)	Gelatin
	MMP-27		59; (–)	-
	MMP-28	Epilysin	56; 45	Casein

Adapted from Woessner & Nagase (2000a), Nabeshima *et al.* (2002) and Visse & Nagase (2003) with additional data from Cole *et al.* (1996), Hanemaaijer *et al.* (1997), Velasco *et al.* (1999), Palosaari *et al*. (2000, 2002), Väänänen *et al.* (2001) and Marchenko *et al*. (2003) and NCBI database (for MMP-27).

a: molecular weight in kilodaltons (proMMP; active MMP).

2.3.1 Collagenases

MMP-1, MMP-8, MMP-13, and MMP-18 (a *Xenopus* collagenase) are capable of degrading native interstitial collagens I, II, and III at a specific site at the 3/4 length from the N-terminus of the molecule (Visse & Nagase 2003). The first matrix metalloproteinase (MMP-1) was a collagenase found in the tadpole tail (Gross & Lapière 1962). MMP-1 is the most ubiquitously expressed collagenase, its expression being low but inducible in a variety of normal cells. Some malignant cells express MMP-1 at high levels constitutively (Birkedal-Hansen *et al.* 1993, Brinckerhoff *et al.* 2000). Of fibrillar collagens, MMP-1 degrades more efficiently type III collagen than either type I or type II (Hasty *et al.* 1987, Mallya *et al.* 1990).

MMP-8 was first found in polymorphonuclear (PMN) leucocytes, which release the enzyme from their intracellular granules (Oronsky *et al.* 1973, Murphy *et al.* 1977). The PMN-type MMP-8 is more glycosylated than the mesenchymal-type MMP-8 found later in chondrocytes (Cole *et al.* 1996), synovial fibroblasts, and endothelial cells, (Hanemaaijer *et al.* 1997), odontoblasts (Palosaari *et al.* 2000) as well as squamous epithelial carcinoma cells (Moilanen *et al.* 2002). MMP-8 prefers type I collagen as a substrate over the types II and III (Hasty *et al.* 1987, Mallya *et al.* 1990).

MMP-13 is a collagenase with a relatively restricted expression profile in normal adult tissues (Freije *et al.* 1994, Brinckerhoff *et al.* 2000, Leeman *et al*. 2002). It was first discovered in breast cancer (Freije *et al.* 1994), but has also been detected in many other malignant neoplasms (Leeman *et al*. 2002) and other pathologic tissues, *e.g.* chronically inflamed gingiva (Uitto *et al.* 1998) and osteoarthritic cartilage (Mitchell *et al.* 1996). Physiological MMP-13 expression is observed in hypertrophic chondrocytes, fibroblasts, and osteoblastic cells of developing fetal bone and postnatally in osteoblasts during endochondral ossification (Johansson *et al.* 1997, Ståhle-Bäckdahl *et al.* 1997), in

fibroblasts of healing gingival wounds (Ravanti *et al.* 1999), and in fetal membranes (Fortunato *et al.* 2003). MMP-13 cleaves type II collagen more efficiently than the types I or III (Knäuper *et al.* 1996). MMP-13 is also a potent gelatinase (Knäuper *et al.* 1996), and exhibits telopeptidase activity (Krane *et al.* 1996, Mitchell *et al.* 1996, Knäuper *et al.* 1997), unlike the other collagenases.

2.3.2 Gelatinases

MMP-2 and MMP-9 were first named type IV collagenases due to their activity against type IV collagen in basement membrane. Later, however, they were renamed as gelatinases due to their efficient activity against denatured collagens, *i.e.* gelatins. (Woessner & Nagase 2000a, Kähäri & Saarialho-Kere 2002.) MMP-2, but not MMP-9, is capable of cleaving even native type I, II, and III collagens into specific 3/4 and 1/4 fragments generated by collagenases (Aimes & Quigley 1995, Konttinen *et al.* 1998a, Patterson *et al.* 2001). Both gelatinases have three repeats of the type II fibronectin domain inserted into the catalytic domain, which bind to native and denatured type I collagen (Murphy *et al.* 1994, Allan *et al.* 1995).

MMP-2 was first found and purified from murine melanoma tissue (Liotta *et al.* 1981, Salo *et al.* 1983). MMP-2 is often constitutively expressed, typically in mesenchymal cells, and its expression profile is wide including many different normal and malignant cells (Birkedal-Hansen *et al.* 1993, Kähäri & Saarialho-Kere 1999). The expression profile of MMP-9 is more restricted. It is found in PMN leucocytes (Sopata & Dancewicz 1974, Murphy *et al.* 1989), stored in secretory granules as monomers, dimers, or covalently bound to lipocalin (Kolkenbrock *et al.* 1996). In other MMP-9-expressing cells, *e.g.* macrophages and keratinocytes, *de novo* synthesis is required for MMP-9 secretion (Woessner & Nagase 2000a, Kähäri & Saarialho-Kere 2002).

2.3.3 Stromelysins

Stromelysin-1 and -2, MMP-3 and MMP-10, are expressed by various normal and malignant cells. They have a similar, rather broad substrate spectrum, which includes many proteoglycans and other ECM molecules. MMP-3 is the more potent of these two, and it also activates several other MMPs (Table 1). MMP-11 is sometimes designated as a stromelysin, but due to differences in sequence and substrate specificity compared to MMP-3, it has also been placed in the category of other MMPs. (Kähäri & Saarialho-Kere 1999, Visse & Nagase 2003.)

2.3.4 Membrane-type matrix metalloproteinases

MT-MMPs are bound to cell plasma membrane by type I transmembrane protein (MT1-, MT2-, MT3-, and MT5-MMP) or by glycosylphosphatidylinositol (GPI) anchor (MT4- and MT6-MMP) (Hernandez-Barrantes *et al.* 2002, Visse & Nagase 2003). Most of them are also found in a shed soluble form. MT-MMPs differ with respect to their physiological and pathological tissue-specific expression profiles and substrate specificities. MT-MMPs are considered important regulators of cell surface and pericellular proteolysis by their own classical ECM-degrading endopeptidases activity, but also by their central role in the activation of other MMPs. (Hernandez-Barrantes *et al.* 2002, Visse & Nagase 2003.)

2.3.5 Matrilysins and unclassified matrix metalloproteinases

Matrilysins (also termed minimal-domain MMPs), MMP-7 and MMP-26, lack the hemopexin domain. Nevertheless, their substrate spectrum, especially that of MMP-7, includes a variety of ECM and other molecules. (Visse & Nagase 2003.) They are both expressed mainly in cells of epithelial origin (Wilson & Matrisian 1996, Marchenko *et al.* 2004).

MMP-12 was first found in alveolar macrophages (Shapiro *et al.* 1993), but it is also physiologically expressed in human fetal hypertrophic cartilage and developing bone (Kerkelä *et al.* 2001). It is capable of cleaving several ECM molecules, including highly stable and resistant elastin (Table 1). MMP-19 was first found in human mammary gland, and it is also expressed in many human adult internal organs (Cossins *et al.* 1996).

MMP-20, enamelysin, has been cloned from human odontoblasts (Llano *et al.* 1997) as well as from porcine and bovine enamel organs (Bartlett *et al.* 1996, Den Besten *et al*. 1998). It is thought to be tooth-specific (Bartlett *et al.* 1996, Llano *et al.* 1997, Grant *et al.* 1999), since in addition to tooth tissues, it has been detected only in cultured human tongue carcinoma cells (Väänänen *et al.* 2001) and some odontogenic tumors (Takata *et al.* 2000, Väänänen *et al.* 2004). MMP-20 expression in enamel-forming ameloblasts is most intense during the early stage of enamel formation (Bartlett *et al.* 1998). MMP-20 processes amelogenin, the major component of the enamel matrix (Llano *et al.* 1997, Fukae *et al.* 1998, Ryu *et al.* 1999), thereby presumably controlling crystallite elongation and the development of the prism structure (Simmer & Hu 2002). The importance of MMP-20 in early enamel development is supported by the severe enamel defects observed in MMP-20-deficient mice (Caterina *et al.* 2002). MMP-20 is first expressed in odontoblasts that become functional and start secreting predentin, and this expression continues after dentin mineralization in fully differentiated odontoblasts (Bègue-Kirn *et al*. 1998, Bourd-Boittin *et al.* 2004, Väänänen *et al.* 2004). In developing teeth MMP-20 expression is observed in pulp cells (Fukae *et al.* 1998, Bourd-Boittin *et al.* 2004), but it is also expressed in the pulp of fully developed teeth (Palosaari *et al.* 2003).

MMP-21, -23, -27, and -28 (current names by NCBI) are quite recently found and currently poorly characterized MMPs (Visse & Nagase 2003).

2.3.6 MMP activation

Most MMPs are secreted as enzyme precursors, zymogens, in which propeptide cysteine binds with its sulfhydryl group to the active-site zinc ion as the fourth ligand constituting the "cysteine switch" (Nagase 1997, Visse & Nagase 2003). *In vitro* the conversion of the proform into a form considered active may be achieved by proteolytic removal of the propeptide, perturbation of the cysteine-zinc interaction, or modification of the sulfhydryl group, allowing interaction of the active-site zinc with a water molecule and exposure of the active site (Springman *et al.* 1990, Van Wart & Birkedal-Hansen 1990). The activation process occurs stepwise in many cases and involves autocatalytic processing. *In vivo* proMMPs activation is observed to localize in the intra- and extracellular milieu and on the cell surface (Nagase 1997, Nagase & Woessner 1999, Visse & Nagase 2003.)

Various host and bacterial proteinases are capable of initiating proteolytic activation by cleaving the proteinase-susceptible 'bait' region in the middle of the propeptide (Sorsa *et al.* 1992, Nagase 1997). This cleavage is followed by further processing of the pro-domain, often by another MMP (Nagase 1997), and MMPs form complexed interaction networks by these activation cascades (Table 1). MT-MMPs are important proMMP activators in the pericellular milieu (Brinckerhoff *et al.* 2000). This type of activation is considered a major mechanism in the regulation of MMP-2 activity, since proMMP-2 is resistant to proteolytic activation by many endopeptidases (Okada *et al.* 1990, Nagase 1997). ProMMP-2 activation by MT1-MMP and MT3-MMP involves interactions with tissue inhibitors of metalloproteinases, with TIMP-2, and with TIMP-2 or TIMP-3, respectively (Strongin *et al.* 1995, Zhao *et al.* 2003). A special type of proteolytic activation, occurring in the intracellular milieu by furin-like serine proteinases of the Golgi apparatus before secretion, was first observed with MMP-11 (Pei & Weis 1995). MMP-21, -23, -28, and MT-MMPs also have this furin recognition sequence (Visse & Nagase 2003).

In vitro proMMPs can be activated by various chemical agents and treatments, including thiol-modifying agents, denaturants, chaotropic agents, reactive oxygens, and heat treatment (Nagase 1997, Visse & Nagase 2003). MMP-2, -8, and -9 can also be activated by acidic pH followed by neutralization (Davis 1991, Tjäderhane *et al.* 1998a).

It used to be postulated that proMMPs are catalytically inactive and require conversion into a lower molecular weight form ("active form") to exhibit function. However, this notion has been recently challenged by the data showing proteolytic activity of specific proMMPs when bound to a substrate or a ligand (Bannikov *et al.* 2002, Fedarko *et al.* 2004).

2.3.7 Inhibition of MMPs

2.3.7.1 Endogenous inhibitors

Four specific tissue inhibitors of MMPs, TIMPs, have been identified in vertebrates (TIMP-1, -2, -3, and -4) (for reviews see Gomez *et al.* 1997, Brew *et al.* 2000, Woessner & Nagase 2000a). TIMPs have molecular weights ranging from 21 to 29 kDa with different glycosylation levels, and they bind to MMPs non-covalently in a ratio of 1:1. TIMPs differ in their gene regulation patterns, tissue-specific gene expression profiles, and binding affinities to each MMP. Most TIMPs inhibit most active MMPs, and some TIMPs also prevent proMMP activation; at least TIMP-1 binds to proMMP-9, and TIMP-2 and -4 bind to proMMP-2. TIMP-3 is found only sequestered to the ECM, whereas other TIMPs are found in a soluble form. Independently of the MMP-inhibitory function, TIMPs are involved in, for instance, the regulation of cell growth, apoptosis, cell morphology, and gonadal steroidogenesis. (Gomez *et al.* 1997, Woessner & Nagase 2000a, Brew *et al.* 2000.)

TIMPs are considered the key MMP inhibitors in tissues, but in body fluids a macroglobulin protein, α_2-macroglobulin, is considered the primary regulator of MMP activity. α_2-macroglobulin is a general endopeptidase inhibitor and an abundant plasma protein. It inhibits MMPs by entrapping the enzyme and sterically hindering the interactions with large MMP substrates. The α_2-macroglobulin-MMP complex is finally encytosed. (Wojtowicz-Praga *et al.* 1997, Visse & Nagase 2003.)

2.3.7.2 Synthetic inhibitors

Most synthetic MMP inhibitors prevent MMP activity by chelating or replacing the active-site zinc ion. MMPs are also inhibited by interaction with the propeptide fragment of an MMP, and some MMP inhibitors may act by coating the substrate and thereby preventing MMP access and activity. (Woessner & Nagase 2000b.)

The MMP-inhibitory effect of tetracycline (TC) and its derivatives was first observed with minocycline, which inhibits the collagenolytic activity of gingival crevicular fluid in the absence of bacteria (Golub *et al.* 1983). Tetracyclines are antibiotics with chelating properties, and they inhibit MMP activity in the extracellular milieu (Golub *et al.* 1998). They also inhibit the oxidative activation of proMMPs by scavenging reactive oxygen species (Lauhio *et al.* 1992, Ramamurthy *et al.* 1993, Golub *et al.* 1994) and possibly promote proMMP proteolysis into inactive forms during activation (Smith *et al.* 1996). Intracellularly TCs down-regulate transcriptional levels of MMPs (Uitto *et al.* 1994, Hanemaaijer *et al.* 1997, 1998b, Sadowski & Steinmeyer 2001). Low-dose doxycycline medication based on MMP inhibition is currently used as adjuvant therapy of adult periodontitis in the United States (Golub *et al.* 1998).

Chemically modified tetracyclines, CMTs, lack the 4-dimethylamino group of the tetracyclines responsible for antibacterial activity (Golub *et al.* 1987, 1998). To date,

several CMTs with different MMP-inhibitory specificity and potency properties have been developed. CMT-8 and CMT-3 are the most potent MMP inhibitors, especially against collagenases. CMT-3 is the only CMT with potency against MMP-1. (Golub *et al.* 1998, Greenwald *et al.* 1998.) CMT-5, a pyrazole analogue of tetracycline, lacks most of the MMP-inhibitory effect of the other CMTs (Golub *et al.* 1998, Greenwald *et al.* 1998), but it does prevent oxidative activation of proMMPs (Sorsa *et al.* 1998).

Bisphosphonates (BPs) are pyrophosphate analogues with high affinity for hydroxyapatite crystals (Fleisch 1998, Widler *et al.* 2002). In humans, they are used to treat conditions involving increased bone resorption, *e.g.* Paget's disease and osteoporosis (Fleisch 1998). BPs impair calcification by inhibiting the dissolution and formation of the calcium phosphate crystals; but their anti-resorptive effect on bone is mediated mainly *via* cellular mechanisms. (Fleisch 1998, Widler *et al.* 2002.) Of the several BP generations, the most recently developed ones, including zoledronate, exhibit high antiresorptive efficacy without extensively inhibiting mineralization in bone. (Widler *et al.* 2002.) In a few reports, BPs have been suggested not to affect MMP synthesis (specifically of cultured carcinoma cells) (Boissier *et al.* 2000) or to affect osteoclasts and osteoblasts without chelating calcium (Evans & Braidman 1994), but a growing body of evidence indicates that BPs are MMP-down-regulating and inhibiting agents with chelating activity (Teronen *et al.* 1997, 1999, Boissier *et al.* 2000, Heikkilä *et al.* 2002, 2003).

Many other molecules also exhibit a wide scale MMP inhibition. For example, hydroxamate-based inhibitors, including Batimastat and Marimastat, which have a collagen-mimicking structure, act by chelating active-site zinc (Wojtowicz-Praga *et al.* 1997). However, with new MMP inhibitors, it may be possible to target only selected MMPs, such as the recently developed synthetic cyclic peptide CTT(HWGFTLC), which seems gelatinase-specific (Koivunen *et al.* 1999).

2.3.8 MMPs and TIMPs in the dentin-pulp complex

MMPs have been detected in both soft and hard tissue compartments of the dentin-pulp complex, and their activity has been suggested to be involved in various physiological processes of the formation and maintenance of the dentin-pulp complex.

During early tooth development, dental mesenchymal cells are observed to express at least MMP-1, -2, -3, -9, and -20 as well as TIMP-1, -2, and -3. The levels of MMP and TIMP-1 expression peak at the time of the beginning of dentin formation in rodents. (Sahlberg *et al.* 1992, 1999, Randall & Hall 2002, Bourd-Boittin *et al.* 2004.) A similar peak of high MMP-2 expression is also observed in newly differentiated dentin-forming odontoblasts of developing human teeth (Heikinheimo & Salo 1995). MT1-MMP has also been observed in odontoblasts of porcine developing teeth (Caron *et al.* 1998).

During primary dentinogenesis odontoblasts secrete at least gelatinases (Betti & Katchburian 1982), proteoglycanases (Fukae *et al.* 1994), and specifically, MMP-3 (Den Besten *et al.* 1989, Hall *et al.* 1999) and TIMP-1 (Hoshino *et al.* 1986) into the zone of intertubular dentin formation, predentin. In predentin MMPs are suggested to be involved

in the organization of the dentin matrix constituents before mineralization (Hall *et al.* 1999, Palosaari *et al.* 2000, Tjäderhane *et al.* 2001a, b). Another site of non-mineralized organic constituents presumably undergoing reorganization before mineralization is inside the dentinal tubules, where at least MMP-3 (Den Besten *et al.* 1989, Hall *et al.* 1999) and TIMP-1 (Hoshino *et al.* 1986) may contribute to intratubular dentin formation.

After the completion of tooth development, physiological dentin formation continues at a slower rate. Odontoblasts of fully developed human teeth synthesize at least MMP-2 and -9 (Tjäderhane *et al.* 1998b), MMP-8 (Palosaari *et al.* 2000), MT1-MMP (Palosaari *et al.* 2002), and MMP-20 (Palosaari *et al.* 2003). These MMPs are also synthesized in human pulp tissue (Palosaari *et al.* 2000, 2002, 2003). The real-time PCR method showed most MMPs, including MMP-2, -10, -11, -14, -15, -16, -20, and -23, to be more intensely expressed in odontoblasts, whereas MMP-13 and -17 were more abundantly expressed in pulp (Palosaari *et al.* 2003). MMP-3 has been observed in odontoblasts of rat incisors (Hall *et al.* 1999) and bovine molars (Den Besten *et al.* 1989), but not in odontoblasts of fully mature human teeth (Palosaari *et al.* 2003). Of the tissue inhibitors of MMPs, TIMP-1, -2, and -3 are expressed in odontoblasts and pulp of fully developed teeth (Hoshino *et al.* 1986, Palosaari *et al.* 2003).

Some of the MMPs involved in dentin formation become embedded into mineralized dentin, since a latent enzyme with collagenolytic activity (Dayan *et al.* 1983, Dumas *et al.* 1985) as well as gelatinolytic metalloproteinases (Fukae *et al.* 1991), specifically MMP-2 (Martin-De Las Heras *et al.* 2000), have been found in mineralized dentin. TIMP-1 is also found in dentin (Geiger & Harper 1981, Hoshino *et al.* 1986, Ishiguro *et al.* 1994). The TIMP-1 concentration follows a decreasing gradient from predentin distally (Ishiguro *et al.* 1994, Goldberg *et al.* 2003), whereas dentinal gelatinases show a reverse gradient (Goldberg *et al.* 2003).

In addition to their physiological roles, the MMPs of the dentin-pulp complex have also been suggested to be involved in pathological processes, including dentin ECM destruction during caries progression (Tjäderhane *et al.* 1998a, 1999), the release and activation of dentinal growth factors due to external non-physiological stimuli (Tjäderhane *et al.* 2001a), the formation of tertiary dentin (Tjäderhane *et al.* 2001a), tissue destruction in pulpal inflammation (Tjäderhane *et al.* 2001a, Gusman *et al.* 2002, Shin *et al.* 2002, Wahlgren *et al.* 2002), and the breakdown of demineralized collagen in the adhesive layer of dental composite restorations (Pashley *et al.* 2004).

2.3.9 MMPs and TIMPs in dental plaque and oral fluids

Dental plaque, a biofilm composed of salivary mucins and microorganisms, contains collagenase (MMP-8) and gelatinases, at least MMP-9 (Sorsa *et al.* 1995). These host MMPs originate from whole saliva, which is a mixture of fluids including saliva from the major and minor salivary glands as well as gingival crevicular fluid (GCF) in dentate individuals. Various gelatinases are present in whole saliva and GCF, MMP-9 being predominant (Ingman *et al.* 1994). The gelatinases originate mainly from GCF (Mäkelä *et al.* 1994, Westerlund *et al.* 1996), even though some MMP-9 may be secreted from the

major salivary glands (Mäkelä *et al.* 1994). Human whole saliva also contains collagenase (Iijima *et al.* 1983), MMP-8, which is predominantly of gingival crevicular fluid PMN cell origin (Sorsa *et al.* 1988, 1990, Uitto *et al.* 1990). In addition to MMPs, whole saliva contains TIMP (Drouin *et al.* 1988).

In some conditions associated with increased caries incidence and activity, salivary MMP levels are increased. In Sjögren's syndrome increased collagenase (Konttinen *et al.* 1994) and MMP-9 activities (Hanemaaijer *et al.* 1998a, Wu *et al.* 1997, Konttinen *et al.* 1998b) are observed in whole saliva. Also, after radiation therapy for head and neck cancer, salivary MMP-9 activity is increased together with decreased salivary pH and flow rate (Vuotila *et al.* 2002).

3 Aims of the study

Even though degradation of the acid-insoluble organic matrix of dentin is an essential process of dentin caries progression, the enzymes responsible for its proteolysis and the exact mechanisms are still unclear. Cariogenic microbes are required for caries development, but the evidence for their collagenolytic or even proteolytic activity is inconclusive.

The matrix metalloproteinase family is mainly responsible for the degradation of the structural macromolecules of the interstitial connective tissues in the extracellular milieu during physiological ECM turnover and reorganization in the human body, presumably including the dentin-pulp complex. As in other tissues, MMP activity may also be altered in pathological conditions of the dentin-pulp complex. MMPs may contribute to the degradation of the dentin organic matrix during caries progression, but also to the response reactions to caries in the dentin-pulp complex. Therefore, these studies were performed to elucidate further the role of host MMPs in dentin caries progression, and to examine the presence and origin of MMPs in carious dentin as well as the changes in MMP expression in the dentin-pulp complex in response to caries.

The following hypotheses and specific aims of the study were set:

1. The degradation of the collagenous organic matrix of dentin during caries progression is an enzymatic process. We hypothesized that host MMPs are involved in this process. Therefore, the effects of MMP inhibitors on dentin caries progression were investigated in the rat *in vivo*. The effects of MMP inhibitors on human salivary gelatinase activity *in vitro* were determined to find out if salivary gelatinases could be involved in the degradation of the dentin organic matrix. (I)
2. Caries elicits response reactions in pulp, and these reactions were hypothesized to involve changes in MMP gene expression. Therefore, the MMP gene expression profiles in the pulp tissue of human sound and carious teeth were examined. MMP-13 was selected for further analysis of mRNA and protein expression in pulp and odontoblasts because of its extremely high level of expression observed in the study. (II)

3. The protein expression of MMP-20 in the dentin-pulp complex soft tissues of sound and carious teeth was examined. The working hypothesis was that this MMP, expressed during tooth formation, is also expressed by the dentin-pulp complex soft tissues of fully developed human teeth, and its protein expression in these tissues changes in response to caries. Since at least some of the MMP-2 expressed during dentin formation is embedded into mineralized dentin, MMP-20 was also hypothesized to be found in dentin. (III)
4. Mineralized dentin contains gelatinases (specifically MMP-2) and as yet uncharacterized collagenases. The aim of the study was to characterize the dentinal collagenases and to study the stability of MMPs in human dentin based on the working hypothesis that the dentinal MMPs are protected by the mineral phase of the tissue. (IV)

4 Materials and methods

4.1 Animal experiment (I)

The protocol for the animal experiment was approved by the Experiment Animal Committee of the Medical Faculty, University of Oulu, Finland. The experiment was performed using 56 female Wistar rats, which were weaned at 21 days of age and randomly divided into six groups. All rats were fed a 41% sucrose diet (Ewos sR36, Brood Stock feed for Rats and Mice, Finnewos Aqua, Turku, Finland) immediately after weaning and throughout the study, and they were inoculated with a fresh suspension of *Streptococcus sobrinus* (ATCC 27351 K 1 Fitzgerald) weekly (Hietala *et al*. 1993) to induce caries. Food and drinking water were freely available, and the rats were weighed weekly to maintain the correct drug dosage and to follow their weight gain.

The experimental drug groups received the following MMP inhibitors: chemically modified tetracycline-3 (CMT-3, Metastat™; Collagenex Pharmaceuticals Inc., Newtown, PA, USA), zoledronate [2-(imidazole-1-yl)-1-hydroxyethane-1,1-bisphosphonic acid (MSD, West Point, PA, USA)], a combination (1:1) of CMT-3 and zoledronate, or a tetracycline derivative lacking most of the MMP-inhibitory effects of TCs, CMT-5 (Collagenex Pharmaceuticals Inc.) (n=8–10 in each group, please see Table 1 in Paper I). The drugs were administered with 1% gelatinous carboxymethyl cellulose (CMC), which allowed reasonable retention of the drug in the oral cavity, followed by swallowing over time. The control groups were administered only the vehicle or nothing. The drugs were given intraorally at the dose of 20 mg *per* kg animal weight on 5 days *per* week for seven weeks.

After the experiment, the animals were decapitated under anesthesia, the mandibles were dissected, defleshed and stored in absolute ethanol. The sizes of the mandibular molar fissure caries lesions were visualized by Schiff's staining, and the areas of dentin caries and total dentin formation were measured as described previously (Larmas & Kortelainen 1989, Hietala *et al*. 1993, Tjäderhane *et al.* 1996). Briefly, the molars were bisected in the mesio-distal direction through the mid-fissures, stained with Schiff's reagent and photographed. The areas of total dentin formation and the areas of caries

lesions were determined from the video image, using computer-based image analyzer (Imaging Technology, Inc., Woburn, MA, USA).

SPSS for Windows Release 6.1 was used for the statistical analyses. The Kolmogorov-Smirnov and Shapiro-Wilks' tests were performed to evaluate if the data met the assumption of normal distribution. Since the data on dentin formation and body weight were normally distributed, analysis of variance (ANOVA) was used to test whether any significant differences existed between the groups; whenever there were differences, Tukey's HSD test was used to determine which groups exhibited a statistically significant difference. With the determinations of dentinal caries, the data did not meet the assumption of normal distribution, which is typical for caries. Therefore, the non-parametric Kruskall-Wallis ANOVA and Mann-Whitney U test were used as described above.

4.2 Human study material (I–IV)

Human teeth (n=225 total), mostly upper and lower third molars from young adults (20–30 years), were obtained for the experiments from the University Student Health Care Center, Oulu, the Department of Oral and Maxillofacial Surgery, University of Oulu, and Oulu Municipal Health Center. The teeth were fully developed with the apex closed, and they were removed as a part of routine treatment (by normal or surgical extraction). Most of the carious teeth were affected by enamel caries or early dentinal caries; no teeth with caries lesions extending into the pulp cavity were included in the study. The teeth were used for the experiments after the patients had given informed consent, and the study protocol for the use of human teeth for experiments was approved by the Ethical Committee of the Northern Ostrobothnia Hospital District. The teeth were immersed in sterile PBS after extraction and used in the experiments immediately or stored at –70°C.

Odontoblast and pulp samples of sound and carious teeth were obtained as previously described (Tjäderhane *et al.* 1998b, Palosaari *et al.* 2000, 2002). Briefly, the crown and root were separated by a horizontal cut with a diamond disc, and the pulp was pulled out of the pulp chamber. The odontoblasts remaining on the walls of the pulp chamber were removed and collected with a dental excavator. The Trizol® extraction protocol (GIBCO-BRL, Gaithersburg, MD, USA) was used to extract the total RNA of the pulps and odontoblasts for cDNA microarray and PCR. The protein phase obtained by Trizol extraction was used for Western blotting. The pulp tissues, odontoblasts, and soft, active carious dentin were also diluted into 1 x Laemmli buffer (Laemmli 1970) for Western blotting. For immunohistochemical staining, sound and carious third molars were bisected longitudinally, fixed in 4 % neutral formalin, demineralized with 0.5 M EDTA, and embedded in paraffin.

Dentinal fluid was collected from an occlusal cavity made with a water-cooled turbine drill through the enamel and 2–4 mm into the dentin of extracted sound human third molars (n=65). The smear layer was removed by etching with 35% phosphoric acid gel (3M Scotchbond 1 Etchant, St. Paul, MN, USA) for 20 s, and the dentinal fluid was collected into 1 x PBS during 20 min incubation at RT. The dentinal fluid samples were

stored at –20°C, pooled (n=3), and concentrated by means of concentration tubes (Millipore Corporation, Bedford, MA, USA) for Western blotting.

Dentin proteins were extracted from sound human molars according to the previously described protocol (Martin-De Las Heras *et al.* 2000) (for a schematic representation of the extraction, see for Fig. 2, p. 59). Dentin proteins were obtained from 13 teeth. To examine the stability and resistance of dentinal MMPs, dentin blocks were steam-autoclaved (134°C for 9 min) once (n=4) or twice (n=5) before crushing and powdering in liquid gas cooling. First, the proteins of non-mineralized compartments were extracted with 4 M guanidine-HCl, 65 mM Tris-HCl (pH 7.4) (G1 extraction fraction), and the dentin powder was then demineralized with 0.5 M EDTA (pH 7.4) (E fractions). After demineralization the guanidine-HCl extraction was repeated (G2 fraction). All steps of protein extraction were performed at 4°C, and the extraction and dialysis solutions contained proteinase inhibitors (2.5 mM benzamidine HCl, 50 mM ε-amino-*n*-caproic acid, 0.5 mM *N*-ethyl maleimide, and 0.3 mM phenylmethylsulphonylfluoride).

Paraffin-stimulated whole saliva was collected from healthy volunteers working in the laboratory and centrifuged (10 000 rpm, 10 min), and the supernatants were used for the assay. The samples were stored at –20°C or analyzed immediately.

4.3 cDNA microarray (II)

cDNA microarray with Atlas Glass Human 1.0 microarrays (Clontech Laboratories, Palo Alto, CA, USA) was used to examine the MMP and TIMP expression profiles of sound (n=18) and carious (n=37) tooth pulps. The chip contained oligonucleotides of 13 MMPs and 4 TIMPs. Due to the large amount of RNA needed for the analysis, the pulps of carious teeth were divided into two pools of 17 and 20, and those of sound teeth into two pools of 8 and 10 for total RNA extraction. 10 µg of RNA of each pool was labeled with fluorescent Cy5 or Cy3 (Amersham Pharmacia Biotech, Buckinghamshire, UK), respectively, with Atlas Glass Fluorescent Labeling Kit (Clontech). Briefly, RNA was reverse-transcribed to cDNA containing aminoallyl dUTP, into which N-hydroxysuccinimide-activated Cy3 or Cy5 were coupled, and the labeled probes were competitively hybridized to the microarray chips by overnight incubation at 50°C. The hybridization was revealed by scanning the chip [GSI Lumonics SA 5000 scanner with ScanArray (GSI Lumonics, Watertown, MA, USA) as scanning software] in the Centre for Biotechnology, Turku, Finland. The images were analyzed with the QuantArray program (GSI Lumonics), and the Cy3/Cy5 ratio of intensities was calculated with Microsoft Excel. For normalization a single scaling factor was applied to each chip, so that the average Cy3/Cy5 ratio was 1.0 (Alizadeh *et al.* 2000). The cut-off point of true expression was set at a level greater than 1.4 (Alizadeh *et al.* 2000), and the cut-off point of the true difference in expression was also set at a level greater than 1.4.

4.4 RT-PCR (II)

The expression of MMP-13 mRNA in pulp and odontoblasts was confirmed with reverse transcriptase-polymerase chain reaction (RT-PCR). The RT and PCR reactions were performed in the same tube as coupled one-step RT-PCR (Aatsinki 1997). At each PCR cycle denaturing temperature was 95°C, while annealing and extension were carried on at 72°C. The primers used for RT-PCR were sense: 5′ AGA TAA GTG CAG CTG TTC AC 3′ and antisense: 5′ TCA TTG ACA GAC CAT GTG TC 3′, and the primers for nested PCR were sense: 5′ AGC ATC TGG AGT AAC CGT 3′ and antisense: 5′ TCA ATG TGG TTC CAG CCA 3′.

4.5 Real-time quantitative PCR (II)

The relative MMP-13 expression levels of individual odontoblast and pulp samples obtained from 6 sound teeth and 9 carious teeth were determined by real-time quantitative PCR performed by the Centre for Biotechnology's TaqMan service (Turku, Finland). The total RNA obtained by the Trizol protocol was treated with one unit of RQ1 Rnase-Free Dnase (Promega, Madison, WI, USA) and further cleaned up with the RNeasy Protect Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. mRNA was then transcribed into cDNA by using one unit of SuperScript II RnaseH Reverse Transcriptase and random hexamer primers (160 µg/ml) (Invitrogen, Carlsbad, CA, USA).

The specific TaqMan primers and probes for MMP-13 mRNA are under the copyright of Applied Biosystems (Assays-on-Demand™, Applied Biosystems). Human GAPDH primers (Medprobe, Oslo, Norway) were used as normalization control in subsequent analysis, in which relative MMP-13 levels were quantified by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). PCR was performed in a total volume of 20 µl with 2 x Absolute QPCR ROX mix (Abgene, Surrey, UK), 300 nM primers, 200 nM probes, and 75 ng of cDNA for GAPDH. For MMP-13 amplification, 125 ng of cDNA and 1 x Assays-on-Demand Gene Expression Assay Mix (Applied Biosystems) containing primers and probes were used. Amplification consisted of 40 cycles of 15 s at 95°C following 1 min at 60°C. Quantification was performed using the ABI Prism® 7700 Sequence Detection System (Applied Biosystems). PCR amplification was performed 4 to 6 times on each sample to reach standard deviation of the threshold cycle (C_T) value less than 0.05. However, in some samples (1 odontoblast and 3 pulp samples) the mRNA yield did not allow more than 5 reactions, and STD of C_T in the range of 0.52–1.41 were obtained for these samples.

4.6 Western blotting (II–IV)

Pulp and odontoblast samples of sound (n=10) and carious (n=14) teeth as well as samples of dentinal fluid (n=3), dentin proteins of sound teeth [including G1, E, and G2 fractions of native (n=13) and autoclaved (n=9) dentin blocks], and soft carious dentin (n=5) were analyzed for the protein expression of MMP-20 and/or collagenolytic enzymes (MMP collagenases, cathepsin K) by Western blotting. The samples were diluted in 1 x Laemmli buffer (with 100 mM DTT for some dentinal samples to reduce disulphide bonds) prepared at 60°C for 20 min and resolved with 12% SDS-PAGE. Before transfer, the gels were stained with 0.5% Coomassie Brilliant Blue to visualize the total protein loaded (Dionisi *et al.* 1995). Then the proteins were transferred on to a nitrocellulose filter (Hoeffer Scientific Instruments, CA, USA) or Immobilon™ P PVDF Transfer membrane (Millipore Corporation, Bedford, MA, USA). Incubation with 5% non-fat dry milk (Difco Laboratories, Detroit, MI, USA or Valio, Helsinki, Finland) in TBS for 1 h was used to inhibit non-specific binding. After washing with TBS-Tween-20, the filter was incubated with primary antibody (Table 2) overnight at RT. Following washings, the filter was incubated with biotinylated secondary antibody and with ABComplex solution (DAKO, A/S, Glostrup, Denmark), including avidin and biotinylated horseradish peroxidase for 1 h. The complexes were detected with the ECL Western Blotting detection kit (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions.

Some filters were reprobed with another antibody as recommended by the manufacturer (Amersham). Briefly, the bound antibodies were removed by incubation in reprobing buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) for 30 min at 60°C. Then the filter was incubated with 5% non-fat milk, and detection was performed as described above.

Table 2. Antibodies used for Western blotting (WB) and immunohistochemical staining (IHC).

Target	Type of antibody	Concentration in WB	Concentration in IHC	Reference
MMP-1	Mouse anti-human	1 µg/ml	-	IM67, Oncogene, Boston, MA, USA
MMP-8	Rabbit anti-human	1 µg/ml	-	(Bergmann *et al.* 1989, Michaelis *et al.* 1990)
MMP-13	Rabbit anti-human	0.7 µg/ml	0.7 µg/ml	Chemicon, Temecula, CA, USA
MMP-13	Mouse anti-human	1 µg/ml	-	IM44L, Oncogene, Boston, MA, USA
MMP-20	Rabbit anti-human	-	0.4–1 µg/ml	Sigma, St. Louis, MO, USA
MMP-20	Mouse anti-human	1 µg/ml	-	Fuji Chemical Industries Ltd., Toyama, Japan
Cathepsin K	Goat anti-human	2 µg/ml	-	SC6507, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA

4.7 Immunohistochemistry (II, III)

The immunohistochemical (IHC) stainings were performed on 6 µm thick deparaffinized tissue sections of demineralized sound (n=4) and carious (n=4) teeth using VECTASTAIN® Elite ABC Kit PK 6101 (Vector Laboratories Inc., Burlingame, CA, USA). Endogenous peroxidase activity was blocked with incubation in 0.3% H_2O_2 in methanol for 3 h. Pretreatment with TBS (pH 9.0) in a microwave oven for 10 min for polyclonal MMP-13 antibody (II) or with 0.4% pepsin for 1 h at 37°C for polyclonal MMP-20 antibody (III) was used to enhance staining. To prevent nonspecific binding, the sections were treated with normal goat serum (diluted 1:20 in 1 x PBS with 2% BSA) for 30 min. Then the sections were incubated with primary antibody (Table 2) overnight at 4°C in a humid chamber. To control the specificity of the staining reactions, sections were incubated with buffer alone or with buffer-diluted normal rabbit serum (1:20 in 2% BSA/PBS) (DAKO, A/S, Glostrup, Denmark) instead of the primary antibody. The sections were incubated with biotinylated anti-rabbit immunoglobulins (DAKO A/S, Glostrup, Denmark) and with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 1 h. AEC (DAKO Corp., Carpinteria, CA, USA) was used as a substrate for peroxidase, and the sections were finally counterstained with Mayer's haematoxylin and mounted in Aquamount (Gurr BDH Chemicals Ltd., Poole, UK).

4.8 MMP-8 immunofluorometric assay (IV)

Total MMP-8 levels in the G1, E1, E3, and G2 extraction fractions of dentin (n=2 for native and n=1 for autoclaved samples) were determined by time-resolved immunofluorometric assay (IFMA). The monoclonal MMP-8-specific antibodies 8708 and 8706 (Medix Biochemica, Kauniainen, Finland) were used as a catching antibody and a tracer antibody, respectively. First, the dentin samples were dissolved in enzyme buffer (50 mM Tris-HCl, 0.2 mM NaCl, 1 mM $CaCl_2$, pH 7.5). 20 µl of samples and 80 µl of assay buffer [20 mM Tris-HCl, 0.5 M NaCl, 5 mM $CaCl_2$, 0.5% BSA, 0.05% NaN_3 and 20 mg/l DTPA, pH 7.5] with 2 µl/ml normal mouse serum were pipetted into the wells. After incubation the wells were washed and filled with 100 µl of assay buffer containing 8706 antibody labeled with europium-chelate (Hemmilä *et al.* 1984). After incubation and washing 100 µl of enhancement solution was added, and 5 min later fluorescence was measured using a 1234 Delfia Research Fluorometer (Wallac, Turku, Finland).

4.9 Gelatin zymography (IV)

Gelatin zymography was used to assess the presence of gelatinases in dentin protein samples (n=9 for native and n=9 for autoclaved samples in all dentin protein fractions). The 1 x Laemmli buffer-diluted samples were electrophoresed into 0.75 mm 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin (Heussen & Dowdle 1980) labeled with MDPF (O'Grady *et al.* 1984). After electrophoresis the gels were washed three times in 0.1% Triton X-100 for 5 min to remove SDS and then incubated in activation solution (50 mM Tris, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, pH 7.5) at 37°C overnight. The gelatinolytic activity in the gels was checked under long-wave UV light, and for storage the gels were stained with 0.5% Coomassie Brilliant Blue, destained, and dried.

4.10 Gelatinase activity assays (I, IV)

Gelatinase activity assay using ^{125}I-labeled gelatin (Risteli & Risteli 1987) as a substrate was performed on saliva samples (n=3) (I) and dentin samples (n=3 for both native and autoclaved samples, including the fractions G1, E2, and G2) (IV) according to a previously published protocol (Mäkelä *et al*. 1994, Tjäderhane *et al*. 1998a). Briefly, saliva samples were incubated for 1 h at 37°C, their pH adjusted with 1 M HCl to pH 4.5-5.0 or 1 M HCl - 8 M NaOH to pH 6 (acid activation) or with 2 mM APMA. Distilled water was added into non-activated samples. For inhibition assays, 5 µl of CMT-3 (200 µg/ml suspended in dH_2O) (Collagenex Pharmaceuticals Inc., Newtown, PA, USA) or distilled water (control samples) was added to 5 µl of saliva and incubated with 100 µl of ^{125}I-labeled gelatin (30 000-50 000 cpm) for 1 h at 37°C. After incubation with gelatin,

20–40% TCA was added in equal volume to each sample to precipitate the undegraded gelatin, and the samples were incubated for 10 min at 4°C. Following centrifugation (10 000 rpm, 10 min at 4°C), half of the supernatant was collected for measurement with a gamma scintillation counter. Lyophilized dentin protein samples were dissolved in enzyme buffer (50 mM Tris, 200 mM NaCl, 1 mM $CaCl_2$, 0.02% NaN_3, 0.05% Brij 35, pH 7.5), incubated with and without 2 mM APMA for 1 h at 37°C, and measured for gelatinase activity as described above.

EnzChek Gelatinase/Collagenase Assay Kit (Molecular Probes, Eugene, OR, USA) was used on dentin samples (n=2 for both sound and autoclaved samples) as recommended by the manufacturer. Briefly, the samples were dissolved in reaction buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.2 mM NaN_3, pH 7.6) and incubated with quenched fluorescein-conjugated gelatin (50 μg/ml) on a microplate at RT. Gelatinolysis was measured at multiple time points with Fluoromax2/Micromax fluorometer (ISA Jobin Yvon-Spex, Edison, NJ, USA).

4.11 Collagenase activity assay (IV)

The collagenase activity of dentin protein samples (n=2) against soluble native type I collagen was assayed by incubating the samples (with and without 1 mM APMA) for 24 h with 1.5 μM native human skin type I collagen in 50 mM Tris-HCl, 0.15 M NaCl, 1 mM $CaCl_2$, pH 7.8. Incubation was terminated by boiling for 3 min, and the type I collagen and its degradation products (αA chains) were separated by 8% SDS-PAGE and visualized by Coomassie Blue staining [the method by Turto *et al.* (1977) modified as Sorsa *et al.* (1992)].

5 Results

5.1 Effects of MMP inhibitors on the rat *in vivo* (I)

5.1.1 Effect on weight gain

In all groups the weights of the rats were within normal limits (National Research Council 1972), and the weight gain trends were similar throughout the experiment.

5.1.2 Effect on total dentin formation

Statistically significant differences in the areas of total dentin formation were observed between the CMT-3 group and the control group (without vehicle inoculation) in first molars, and between the CMT-3+zoledronate group and the control group (with vehicle inoculation) in third molars, but the effects of the MMP inhibitors on total dentin formation were not consistent (Table 3).

Table 3. Total dentin formation. Total dentin area as mean (95% confidence intervals) in $\mu m^2 \times 10^3$ under the central fissure of each molar.

Group	1st molar	2nd molar	3rd molar
Control	378 (335; 422)*	291 (260; 323)	254 (223; 285)
Control+CMC	365 (339; 391)	268 (248; 289)	224 (200; 249)*
CMT-3	316 (290; 341)*	262 (247; 277)	254 (237; 271)
Zoledronate	338 (302; 374)	273 (252; 295)	260 (242; 277)
CMT-3+zoledronate	343 (307; 380)	288 (264; 312)	271 (247; 295)*
CMT-5	329 (304; 353)	249 (229; 270)	241 (221; 260)

*: significant difference ($p<0.05$) between the groups by Tukey's HSD test.

5.1.3 Effect on dentin caries progression

The MMP inhibitor CMT-3 alone demonstrated the most pronounced dentin caries reducing effect on rat mandibular molars (Fig. 1 A–C). Zoledronate and the combination of CMT-3 and zoledronate also reduced dentin caries progression. The mean areas of dentin caries lesions were significantly larger in one or both control groups than in the CMT-3, zoledronate and CMT-3+zoledronate groups in all molars ($p<0.05$; Kruskall-Wallis ANOVA with Mann-Whitney U test). A significant difference was also observed between the CMT-5 group and one control group in second and third molars.

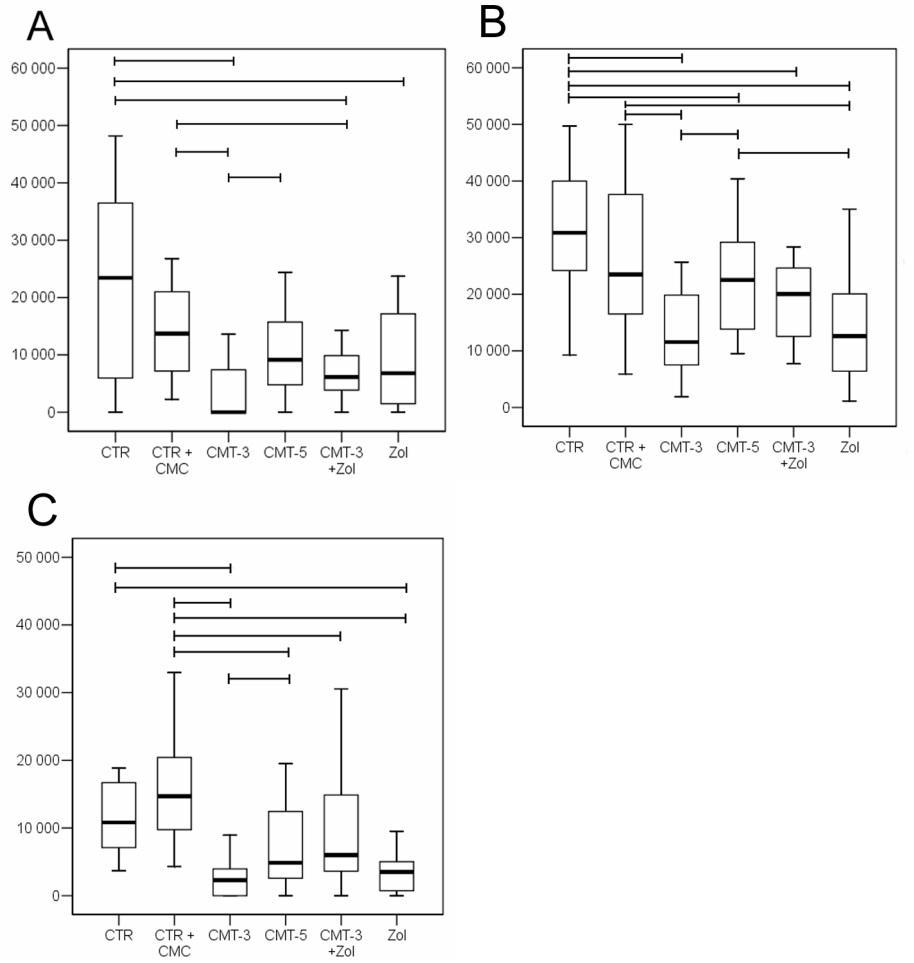

Fig. 1. The area of dentin caries (μm^2) as revealed by Schiff's staining in rat mandibular first (A), second (B) and third (C) molars. The boxes show the 1st and 3rd quartiles with the median value inside, and the whiskers show the maximum and minimum. The groups connected with a line differ significantly ($p<0.05$) by Mann-Whitney U test. Abbreviations: CTR, control group not receiving vehicle (1% CMC); CTR+CMC, control group receiving vehicle; CMT: chemically modified tetracycline; Zol: zoledronate.

5.2 Inhibition of human salivary MMPs by CMT-3 *in vitro* (I)

CMT-3 inhibited both APMA and acid-activated human salivary gelatinases in gelatinase activity assay. The saliva sample treated with an optimal MMP activator APMA served as the reference value (100%). Acid activation at pHs of 4.5, 5.0, and 6.0, followed by neutralization, caused salivary gelatinase activity to reach 84%, 34%, and 12% of the reference value, respectively. Addition of CMT-3 before activation inhibited the activity of the APMA-treated sample by 92%. The activity of acid-treated samples at pHs of 4.5, 5.0, and 6.0 was inhibited by CMT-3 by 83%, 98%, and 100%, respectively.

5.3 MMP and TIMP expression in the dentin-pulp complex soft tissues of sound and carious human teeth (II, III)

5.3.1 MMP and TIMP mRNA expression in pulp (II)

By cDNA microarray, the most intense signal was detected for the expression of MMP-13 mRNA in pooled pulp samples of carious and sound teeth, the signal being over 10-fold compared to that of other positive MMPs or TIMPs. MMP-16 (MT3-MMP) was positive in both carious tooth samples and in the first set of sound tooth samples, and MMP-12 was detected in sound and carious tooth samples of the first array. TIMP-1 was positive in all samples, whereas TIMP-2 and -4 were only detected in the carious tooth pulps of the first array.

With cDNA microarray, no definite differences were detectable between the sound and carious tooth samples. A comparison of the first pools of sound and carious tooth pulps showed MMP-12, MMP-13, and MMP-16 to be up-regulated in the carious tooth samples by more than 1.4 times, while TIMP-1 was down-regulated by more than 1.4 times. However, no changes were detected in the second set of cDNA microarray.

5.3.2 MMP-13 mRNA expression in pulp and odontoblasts (II)

RT-PCR confirmed the expression of MMP-13 mRNA in the pulp and odontoblast samples of sound and carious teeth. The relative MMP-13 levels of individual samples were further studied by real-time PCR, using GAPDH as an endogenous control gene. In general, the interindividual variation between pulp samples was high (range 0.5–668% of the mean level of MMP-13 among all samples), and while that between odontoblast samples was lower (range 3–46% of the mean level of MMP-13 among all samples). When all samples were included in the analysis, the mean MMP-13 levels were 35% up-regulated in the pulps of enamel caries teeth, but 77% down-regulated in the pulps of dentin caries teeth compared with sound teeth pulps. However, when samples with

insufficient repeats of PCR (STD of C_T>0.05) were excluded from the analysis, the MMP-13 levels were down-regulated in the pulps of teeth with both enamel and dentin caries.

In odontoblasts a decreasing trend of MMP-13 mRNA expression with more advanced caries was observed both in an analysis of all samples and when one insufficient sample was excluded. The average MMP-13 expression was 3.9- to 5.7-fold in the sound teeth pulps compared to the odontoblasts of sound teeth.

5.3.3 MMP-13 protein expression and distribution pattern in pulp and odontoblasts (II)

By Western blotting MMP-13 protein expression was observed in odontoblasts and pulp samples as major bands of 60 and 48–50 kDa corresponding to latent and active MMP-13. Immunohistochemical staining revealed MMP-13 immunoreactivity in some odontoblasts and pulpal fibroblasts.

A comparison of MMP-13 protein levels in relation to total protein loaded by Western blotting failed to show any major differences between the pulps or odontoblasts of sound and carious teeth. The IHC staining pattern with MMP-13 antibody was also similar in sound and carious teeth.

5.3.4 MMP-20 protein expression and distribution pattern in pulp and odontoblasts (III)

The protein expression of MMP-20 was detected in pulp and odontoblasts by Western blotting as a major double band of 46 and 43 kDa corresponding to the active form of MMP-20. Several higher and lower molecular weight bands were also detected. The 46 kDa band of MMP-20 was also observed in dentinal fluid, where an occasional 57 kDa band corresponding to proMMP-20 was also detected. IHC revealed MMP-20 in the odontoblastic layer, with an increase in the intensity of the color reaction due to the increasing frequency of stained odontoblasts towards radicular dentin. At the cellular level, the staining was most intense in the odontoblast cell body, especially in its proximal part. Positive staining for MMP-20 was also detected in predentin as well as in some pulpal fibroblasts and endothelial cells.

No marked differences in MMP-20 protein levels in relation to the total protein loaded were detectable between the odontoblasts or pulps of sound and carious teeth by Western blotting. No differences became evident between the IHC staining patterns of the dentin-pulp complex soft tissues of sound and carious teeth, either.

5.4 MMPs in human dentin (III, IV)

5.4.1 Presence of MMPs in sound dentin (III, IV)

MMP-20 was detected by Western blotting in EDTA extracts of dentin and second guanidine extracts (G2) as a major double band of 46 and 43 kDa corresponding to active MMP-20 with several bands of lower molecular weight (III). The G1 fraction was negative for MMP-20. (Table 4.)

MMP-8 immunoreactivity was observed in the G1, E, and G2 extracts by Western blotting (IV). In non-reduced dentin samples MMP-8 was detected in a complexed form (molecular weight above 100 kDa), whereas bands of 50 and 62 kDa were present in samples reduced with DTT. The presence of MMP-8 in dentin was confirmed by IFMA, which demonstrated a decreasing gradient of total MMP-8 with further steps of the extraction protocol (Table 4). Dentinal samples were negative for MMP-1 and -13 as well as cathepsin K by Western blotting.

Gelatinases were detected by zymography in the G1, E, and G2 fractions of dentin as a major band of 59 kDa corresponding to the active form of MMP-2 with several lower molecular weight bands (IV). In some samples, mostly of G1 fractions, a band of 66 kDa corresponding to proMMP-2 was observed.

The dentin protein samples of autoclaved teeth were negative for MMP-8 and MMP-20 by Western blotting and MMP-8-IFMA (IV). In gelatin zymography faint low molecular weight bands of 20–30 kDa could be observed in the E and G2 fractions of some autoclaved teeth, and a faint 59 kDa band was detected in some G2 fractions.

5.4.2 Collagenase and gelatinase activity of sound dentin (IV)

Dentin samples (G1 and E1 fractions) exhibited vertebrate collagenase activity against soluble type I collagen *in vitro* by the generation of the characteristic 3/4 (αA) cleavage products. Type I collagenase activity was not affected by the MMP activator APMA.

Gelatinase activity was detected in all protein fractions of native dentin by gelatinase activity assays using ^{125}I-labeled and fluorescein-conjugated gelatin as substrate (Table 4). Gelatinase activity was greatest in the G2 fraction. In both gelatinase activity assays the dentin protein samples of autoclaved teeth demonstrated gelatinase activity in the E2 and G2 fractions, but not in the G1 fraction. Pretreatment with APMA reduced gelatinase activity in all groups showing some activity.

Table 4. Distribution of MMP-8, MMP-20 and gelatinases in the different dentin protein fractions of native and autoclaved teeth.

	Non-mineralized matrix (G1)		Mineral-bound proteins (E)		Mineralized organic matrix (G2)	
MMPs	Native	Autoclaved	Native	Autoclaved	Native	Autoclaved
MMP-8	+++	-	++	-	+	-
MMP-20*	-	-	+	-	+	-
Gelatinases	+	-	++	+	+++	++

The presence of MMP-8 and MMP-20 in human dentin was examined by Western blotting and MMP-8-IFMA. The presence and activity of dentinal gelatinases was examined by gelatin zymography and gelatinase activity assays. Relative MMP level (MMP-8) or MMP activity (gelatinases) is expressed by plusses (+: lowest level of enzyme or activity; +++: highest level or activity of enzyme)

*: MMP-20 levels not quantified

5.4.3 MMP-20 in carious dentin (III)

In IHC with MMP-20 antibody some dilated dentinal tubules were positively stained in carious teeth. Western blotting showed occasionally a faint band of 43 kDa corresponding to active MMP-20 in soft carious dentin samples.

6 Discussion

6.1 Effect of MMP inhibitors on dentin caries progression *in vivo* (I)

In the present study, the MMP inhibitors zoledronate and CMT-3 decreased the area of dentin caries lesions significantly in rat mandibular molars, suggesting the involvement of host MMP activity in dentin caries progression. A slight decrease in the area of dentin caries lesions was also observed with CMT-5, which is an essentially ineffective inhibitor of active MMPs, but capable of preventing the oxidative activation of proMMPs (Golub *et al.* 1998, Greenwald *et al.* 1998, Sorsa *et al.* 1998). This might imply some oxidative proMMP activation in dentinal lesions during caries progression.

The oral administration of the gelatinous test mixtures was likely to result in both local and systemic effects of MMP inhibitors on host MMPs, since the test mixture remained in the oral cavity after delivery, but was eventually swallowed. Carious dentin in the rat mandibular molars was visualized by Schiff's staining, which reveals more advanced dentin caries (Hietala *et al.* 1993), possibly with involvement of proteolysis (König *et al.* 1958), than the fluorescence method, which was used in a previous experiment with an MMP inhibitor (Tjäderhane *et al.* 1999). As shown previously by the dentin fluorescence method, CMT-3 inhibits early dentin demineralization during caries progression significantly only in rat mandibular third molars (Tjäderhane *et al.* 1999). In mandibular third molars primary dentin was still synthesized during the experiment (Shellis & Berkovitz 1981), which seems to suggest a systemic effect of CMT-3 inhibition on the early dentin caries process. For example, it is possible that the tetracycline derivative CMT-3 may have accumulated into forming dentin. On the other hand, the reducing effect of MMP inhibitors on more advanced lesions was similar in all mandibular molars (I), suggesting the involvement of local effects and/or systemic effects *via* odontoblastic/pulpal MMPs. To elucidate the possible differences in the effects of CMT-3 on dentin caries progression between the two methods, a research design with different lengths and initiation times of caries induction and different methods of caries visualization could be used in the future.

The decreased dentin caries progression may have resulted from the inhibition of MMPs of different origins, including saliva, dentin, or dentinal fluid (odontoblasts and

possibly even pulp), combined or alone. Basically, the discrimination between the local and systemic effects of MMP inhibitors on dentin caries progression could be examined in rats, *e.g.* by the administration of MMP inhibitor *via* a gastric tube or a subcutaneous injection, eliminating most of the effect on salivary MMPs. However, at least in humans, systemic administration of doxycycline decreases salivary total and active MMP-8 levels, without affecting salivary MMP-9 levels (Lauhio *et al.* 1994a, 1994b, Nordström *et al.* 1998). Therefore, the exclusive systemic effect of tetracyclines on host MMPs may not be achieved. On the other hand, the effects of odontoblastic and pulpal MMPs could be excluded by eliminating the soft tissues of the dentin-pulp complex. However, dentinal MMPs are included in the experimental design if dentin ECM is used as the substrate. Therefore, combinations of different approaches are required to elucidate and confirm the significance of host MMPs of different origins in dentin caries lesion progression.

The animal study was designed to examine the effects of the inhibition of host MMPs on dentin caries progression, but there are possible additional mechanisms of action, which would require further studies. For example, even though the antiresorptive effect of bisphosphonates on bone is exerted mainly by cellular effects on osteoclasts, BPs have direct effects on minerals by inhibiting the formation of calcium phosphate crystals and delaying the dissolution of these crystals (Fleisch 1998, Widler *et al.* 2002). Therefore, the direct effect of zoledronate on dentin demineralization in this experiment (I) cannot be ruled out.

Also, some chemically modified tetracyclines, including CMT-3, but not CMT-5, have recently been shown to exhibit fungistatic and fungicidic activity (Liu *et al.* 2002) instead of antibacterial effects (Golub *et al.* 1987, 1998). For example, *Candida albicans,* which is considered a cariogenic microbe (van Houte 1980), showed the greatest sensitivity to the CMT-3 of the yeasts tested, the growth being inhibited by concentrations well below the CMT-3 concentration of the test mixture used in the study (I) (Liu *et al.* 2002). In comprehensive reviews, however, BPs have not been reported to have antifungal properties (Fleisch 1998, Widler *et al.* 2002). Since zoledronate also decreased dentin caries progression in rats, it seems unlikely that the caries-reducing effect of MMP inhibitors would have involved any significant antifungal effects. Nevertheless, this possibility should be evaluated in caries experiments using CMTs *in vivo* in the future.

In theory, the antiproteolytic effects of MMP inhibitors could have been mediated *via* bacterial proteases in addition to host MMPs. Previously, S*treptococcus mutans* was shown in one report to possess a gelatinase with metalloprotease characteristics, *i.e.* enzyme inhibition by chelation (Jackson *et al.* 1997). However, there are several publications reporting no collagenolytic or even gelatinolytic activity by *Streptococcus mutans* (Tjäderhane *et al.* 1998a, Dung & Liu 1999) or no activity against dentin collagen (van Strijp *et al.* 1997). Also, the evidence for the collagenolytic potency of cariogenic bacteria in general is weak (*e.g.* Katz *et al.* 1987, van Strijp *et al.* 1994, 1997, Tjäderhane *et al.* 1998a). Therefore, at present, the findings do not support the conclusion that the primary target of MMP inhibitors was bacterial proteases, even though this possibility cannot be excluded. For future experiments, the issue of host vs. microbial enzymes poses a challenge, since in *in vivo* experiments with laboratory animals bacteria are essential to induce caries.

Rodents and humans differ with respect to their complete genomic repertoire of proteases, including the MMP family. For example, mice lack the MMP-26 that is present

in humans, and they have two orthologue genes to human MMP-1. (Puente *et al.* 2003.) Furthermore, there are differences in the expression profiles of MMPs between humans and rodents, *e.g.* the enzyme corresponding to human interstitial collagenase MMP-1 is MMP-13 in rodents. Therefore, even though animal experiments provide an opportunity to study the effects of MMP inhibition *in vivo,* it is important to examine human material to obtain specific information on the expression and possible roles of MMPs in humans.

6.2 Effect of MMP inhibitors on salivary gelatinases (I)

Acidic pH followed by neutralization is a typical sequence in a caries lesion after the ingestion of fermentable carbohydrates. Purified gelatinases (MMP-2 and -9) and MMP-8 as well as human salivary MMPs are activated by such pH changes ("acid activation") (Davis 1991, Tjäderhane *et al.* 1998a). Also, the dentin organic matrix is degraded by acid-activated salivary proteases *in vitro*. Therefore, host salivary MMPs have been suggested to contribute to dentin caries progression (Tjäderhane *et al.* 1998a). However, acid activation may not be limited to salivary MMPs. For example, the progelatinases (proMMP-2 and -9) detected in carious human dentin (Tjäderhane *et al.* 1998a) are also found in sound mineralized dentin (at least proMMP-2; Martin-De Las Heras *et al.* 2000) and synthesized by odontoblasts (Tjäderhane *et al.* 1998b). Nevertheless, CMT-3 inhibited salivary gelatinase activity *in vitro*, and the inhibition of salivary gelatinases is thus one possible explanation for the reduced dentin caries progression in rats.

In a recent study, no correlation between salivary gelatinase activity and dentin organic matrix degradation *in situ* was found (van Strijp *et al.* 2003). However, totally demineralized dentin slabs were used in these experiments, which are more susceptible to proteases than true carious dentin (Prophet & Atkinson 1953, Konetzka *et al.* 1956, Armstrong 1958, Young & Massler 1963), and their susceptibility to bacterial non-specific proteases may be increased. On the other hand, the filtered human whole saliva used in the experiments with CMT-3 contains proteolytic enzymes of both host and microbial origin. In summary, the findings on the effects and significance of salivary MMPs on dentin caries progression are controversial, and no final conclusions concerning their role in dentin caries progression can therefore be drawn at the present.

6.3 Expression of MMPs and TIMPs in the pulp of sound and carious human teeth (II, III)

The pulp is heterogeneous soft tissue with various cell types as well as neural and vessel components embedded in the extracellular matrix. Similarly to other soft connective tissues, pulp involves ongoing ECM turnover, and pulpal MMPs have been suggested to contribute to this physiological process (Palosaari *et al.* 2000, Tjäderhane *et al.* 2001a). A caries attack causes many changes in the pulp, *e.g.* infiltration of inflammatory cells

(Brännström & Lind 1965) and accumulation of pulpal cells into the cell-free zone (Björndal *et al.* 1998). In the early phase of dentinal caries the metabolic activity of pulp cells increases (Kobayashi *et al.* 1996). Due to injury, interodontoblastic junctions may be impaired (Turner *et al.* 1989), whereupon pulpal proteins, even those originating from plasma (Knutsson *et al.* 1994), leak into dentin tubules and presumably also into carious dentin. In the case of severe injuries, PMN cells may also penetrate into dentinal tubules (Bergenholtz 1977). The findings on changes in MMP synthesis in advanced pulpal inflammation have been conflicting, as the levels of MMP-2 and -3, for example, have been reported to be both increased (Shin *et al.* 2002) and decreased (Gusman *et al.* 2002) in heavily inflamed pulp.

In study II, the MMP and TIMP mRNA profiles of pooled pulp samples of sound teeth and teeth with enamel or early dentinal caries were examined by cDNA microarray. The cDNA microarray method cannot be considered strictly quantitative, since the content of cytidine (C) and guanosine (G) nucleotides, which have higher affinity towards each other than adenosine (A) and thymidine (T), influences the affinity of the probe to the oligonucleotide. Even with this limitation in mind, cDNA microarray demonstrated a high level of expression of MMP-13 in all pooled pulp samples. Expression of TIMP-1, albeit at a lower level, was demonstrated in all pulp samples, and the expression of MMP-16 (MT3-MMP), MMP-12 as well as TIMP-2 and -4 in some pooled pulp samples.

Since MMP-13 expression was consistently highest among MMPs and TIMPs in the pulp, its mRNA and protein expression and localization were further studied. Real-time quantitative PCR revealed very high variation in the relative MMP-13 mRNA levels between pulp samples from individual sound teeth and teeth with enamel caries (II). Differences in lesion activity may explain some of the high variability between the pulps from teeth with enamel caries. However, the similar developmental stage of the pulps of sound teeth leaves the reasons for the wide range of physiological MMP-13 expression obscure. The variation in MMP-13 expression levels between the pulps of teeth with dentinal caries was far less pronounced, but the group sample size was also smaller.

The low mRNA yield of some individual pulp samples did not allow a sufficient number of reactions to be run with real-time PCR (II). The raw data suggested the possibility of differential expression of pulpal MMP-13 mRNA in teeth with initial and deeper caries, which could explain the up-regulation of MMP-13 observed in carious tooth pulps in the first set of cDNA microarrays, which was absent from the second set of arrays. However, the data analysis with the exclusion of insufficient samples did not support this conclusion. In the future, studies with larger sample size need to be conducted before final conclusions on the regulation of pulpal MMP-13 expression in response to caries can be drawn. However, it should be noted that the level of expression of an MMP does not alone dictate its biological activity, which is also influenced by the level of translation and by extracellular activating and inhibiting factors.

The presence of MMP-13 protein in the pulp was confirmed by Western blotting (II). Even though the level of gene expression of MMP-13 was very high in pulp, pulpal fibroblasts were not uniformly stained in immunohistochemical stainings, and the color reaction of the stained cells was not very intense. Various technical matters affect the intensity of the color reaction in IHC, which is not a quantitative method for protein detection. However, a true difference in the levels of gene expression and the presence of the protein product, due to the regulation of synthesis or protein turnover, for instance,

could also explain the discrepancy between the high level of MMP-13 expression in cDNA microarray and the moderate/faint IHC staining.

MMP-13 is expressed at low levels or is absent in most normal adult tissues (Freije *et al.* 1994, Brinckerhoff *et al.* 2000, Leeman *et al.* 2002). Tissues with physiological MMP-13 expression include fetal membranes (Fortunato *et al.* 2003), fetal bone, and sites of postnatal endochondral ossification (Johansson *et al.* 1997, Ståhle-Bäckdahl *et al.* 1997) and healing gingival wounds (Ravanti *et al.* 1999). Of these, wounded gingiva and bone during intramembranous ossification are sites where MMP-13 expression is detected in fibroblastic cells. The most common fibroblast collagenase, MMP-1, is expressed in cultured pulpal fibroblasts (Tamura *et al.* 1996, Lin *et al.* 2001), but undetectable or expressed at a very low level in normal native pulp tissue (Gusman *et al.* 2002, Shin *et al.* 2002, Palosaari *et al.* 2003). Therefore, the data suggests that MMP-13, but not MMP-1, is the key collagenase in human pulp, a feature that seems quite exceptional among normal adult tissues. Even though MMP-13 prefers type II collagen as a substrate over the types I and III (Knäuper *et al.* 1996), it is by no means inactive against these major pulpal collagens (Tsuzaki *et al.* 1990). Other functions of MMP-13, including gelatinase (Knäuper *et al.* 1996) and telopeptidase activity (Krane *et al.* 1996, Mitchell *et al.* 1996, Knäuper *et al.* 1997) as well as the processing of other ECM and non-ECM molecules (Leeman *et al.* 2002), could also be of importance in pulp.

The expression of MMP-16 (MT3-MMP) and MMP-12 was detected in pulp with cDNA microarray (II). Previously, MMP-16 and -12 have not been detected in pulp by real-time PCR (Palosaari *et al.* 2003), and their protein expression in pulp therefore needs to be confirmed.

The MMP-20 gene was not included in the cDNA microarray chip, but its protein expression was observed in the whole pulp tissue by Western blotting, and in fibroblasts and endothelial cells by immunohistochemical staining (III). No major differences in MMP-20 protein expression could be detected between sound and carious teeth pulps with either method. Also, IHC showed MMP-20 immunoreactivity more uniformly in odontoblasts than in pulpal fibroblasts, which were stained sporadically. This is consistent with the previous findings of real-time PCR showing a lower level of MMP-20 in pulp than odontoblasts (Palosaari *et al.* 2003). MMP-20 expression in tissues or cells of non-dental origin is very rare (Grant *et al.* 1999, Väänänen *et al.* 2001). Therefore, it is possible that the expression of MMP-20 in pulp, especially in fibroblast-like cells, reflects the possible capability of these cells to differentiate into mineralized connective tissue forming cells. This assumption is further supported by the abundant co-distribution of MMP-13 (II) and its potential substrate tenascin-C (Knäuper *et al.* 1997) in pulp (Lukinmaa *et al.* 1991, 1996). Tenascin-C is an ECM glycoprotein with restricted distribution in normal adult tissues, similarly to MMP-13, and it has also been suggested to serve as a molecular marker for cells with potential to differentiate into cells with mineralized tissue formation (Lukinmaa *et al.* 1991, 1996).

6.4 Odontoblastic MMPs in sound and carious human teeth (II, III)

Odontoblasts are the first cells to encounter the caries stimulus, and they are thought to have an important role in the dentin-pulp complex repair and defense reactions by forming intratubular and tertiary dentin (Smith 2002). In some limited early studies on humans (Feldman & Lefkowitz 1942) and rats (Brown & Lefkowitz 1966) reduced caries incidence or progression was observed in teeth devoid of the cellular components of the dentin-pulp complex. Later, it was demonstrated in rats that the removal of pulpal soft tissues decreases caries susceptibility significantly during a high-sucrose diet (Steinman *et al.* 1980). The factors favoring caries progression in teeth with dentin-pulp complex soft tissues left may include nutritive factors for cariogenic bacteria, but also undefined disturbed pulpal function (Steinman *et al.* 1980). This pulpal dysfunction might also include increased secretion and/or activation of odontoblastic, and possibly also pulpal, MMPs. In these studies, the differences in MMP-13 mRNA and protein (II) as well as MMP-20 protein (III) expression in odontoblasts of sound and carious teeth were examined.

MMP-20 has an important role in enamel formation, since MMP-20-deficient mice have teeth with thin delaminating enamel (Caterina *et al.* 2002). MMP-20 is also expressed during primary dentin formation in odontoblasts (Bègue-Kirn *et al*. 1998, Bourd-Boittin *et al.* 2004, Väänänen *et al.* 2004). The MMP-20-positive staining in both the predentin zone and dentinal fluid (III) suggests that MMP-20 has a role in both inter- and intratubular dentin formation. Also, the increasing gradient of MMP-20-positive odontoblasts in the apical direction (III) suggests that the odontoblastic MMP-20 expression level reflects the rate of physiological dentin formation. To date, however, no dentinal defects have been reported in MMP-20 knockout mice. This may indicate that whatever the role of MMP-20 in odontoblasts may be, in the absence of its expression other MMPs may take over the task.

IHC showed the MMP-20 protein to be localized in the odontoblast cell body, with weaker staining in the processus (III). Interestingly, similar localization of MMP-20 in rat incisive odontoblasts with continually active dentin formation (Bourd-Boittin *et al.* 2004), but also of gelatinases (Goldberg *et al.* 2003) and MMP-3 (Hall *et al.* 1999), has been detected by immunoelectron microscopy. This type of cellular localization would suggest the secretion of MMP-20 mainly in proximal predentin and, therefore, possibly MMP-20 activity in the early events of intertubular dentin formation in predentin (Linde & Goldberg 1993).

Amelogenin, a major protein of developing enamel, is a substrate for MMP-20 *in vivo* (Ryu *et al.* 1999, Caterina *et al.* 2002). In odontoblasts, amelogenin expression is highest during mantle dentin formation and considerably lower in fully differentiated odontoblasts (Oida *et al.* 2002, Papagerakis *et al.* 2003, Bourd-Boittin *et al.* 2004). Interestingly, dentin contains amelogenin-derived peptides possessing chondrogenic and osteogenic properties (Nebgen *et al.* 1999), which could be hypothesized to have a signaling role for the dentin-pulp complex repair processes due to dentinal injury. These amelogenin-derived peptides result, at least in part, from the alternatively spliced amelogenin mRNA found in odontoblasts (Veis *et al.* 2000), but odontoblastic MMP-20 may also contribute to amelogenin processing. The actual role of MMP-20 in

odontoblasts remains obscure, even though, in fully developed teeth, odontoblasts are the main cellular source of MMP-20 (Palosaari *et al.* 2003, III).

No major differences in MMP-20 protein expression between odontoblasts of sound and carious teeth could be detected (III). The protein levels of MMP-13 were also similar in pooled sound and carious tooth odontoblasts, but real-time quantitative PCR showed the mean MMP-13 mRNA levels to be down-regulated in odontoblasts of carious teeth, especially with dentin caries (II). It is possible that pooling of the odontoblasts masks some differences in mRNA and/or protein expression. Nevertheless, MMP-13 seems to be a minor product of odontoblasts, as supported by the finding of a lower level of expression compared to pulp (Palosaari *et al.* 2003, II). In addition, no immunoreactivity of MMP-13 could be detected in human dentin (IV). In the future, it would be interesting to examine the possible differences in other odontoblastic MMPs in response to caries, because different MMPs may be regulated in distinct ways. For example, the growth factor TGF-β1 down-regulates MMP-8 expression (Palosaari *et al.* 2000) but up-regulates MMP-9 expression in odontoblasts (Tjäderhane *et al.* 1998b, Palosaari *et al.* 2003), whereas MMP-20 levels are not markedly altered by TGF-β1 (Palosaari *et al.* 2003). Therefore, the studies on possible MMP expression changes in odontoblasts at the different phases of caries progression, *e.g.* by cDNA microarray should be performed in the future.

The yield of proteins and particularly mRNA of odontoblasts in an individual tooth is low, especially in carious teeth (V. Pääkkönen, personal communication). Therefore, to date, pooling of odontoblastic samples has been necessary for most applications. Changes in the odontoblastic layer in response to caries or other external stimuli are observed first only in the odontoblasts beneath the stimulus. Also, the stage and activity of the caries lesions may affect the odontoblastic response, as observed previously in cellular changes (Magloire *et al.* 1992, Björndal *et al.* 1998). The collection of such a small cell population is possible by *e.g.* laser capture microdissection (Emmert-Buck *et al.* 1996), but further quantitative analysis of the extremely small mRNA amounts requires very sensitive methods. *In situ* hybridization as a semiquantitative method could be a useful tool in gaining information on gene expression changes in affected odontoblasts.

In murine tooth germ culture increased predentin width, impaired dentin mineralization, and altered proteoglycan processing have been observed in response to the MMP inhibitors Marimastat and CT_{1166} (Fanchon *et al.* 2004), which suggests that MMPs have a crucial role in dentin formation. Also, bisphosphonates have been observed to disturb both dentin organic matrix formation and dentin mineralization (van den Bos & Beertsen 1987, Ogawa *et al.* 1989), but contrariwise, to accelerate root dentin formation *in vitro* (Sommercorn *et al.* 2000). Therefore, we measured total dentin deposition in our *in vivo* experiment with rats (I) to monitor the odontoblastic function. The mandibular first and second molars had erupted and reached functional occlusion during the first week of the experiment (Shellis & Berkovitz 1981), after which the rate of dentinogenesis has been suggested to slow down (Johannessen 1961, Kortelainen & Larmas 1994). On the other hand, in mandibular third molars crown formation had just been completed when the experiment started (Shellis & Berkovitz 1981), and primary dentinogenesis with rapid dentin formation was still going on. However, we did not detect congruent influences of either MMP inhibitor on total dentin formation in any molars (I). In parenteral administration a dose of 10 mg/kg of BPs has been suppressive

on dentin mineralization in rats, but the bioavailability of the most newly developed BPs with oral delivery is below 1% (Fleish 1998), making systemic effects of BPs on mineralization in this experiment rather unlikely.

The method of measuring total dentin formation has previously revealed *e.g.* the disturbing effect of a 10% xylitol diet on the molars of older rats than those used in this study (I), with an even slower rate of dentin formation (Tjäderhane *et al.* 1996), but total dentin formation is obviously not an equally sensitive indicator as the dentin formed during the experiment, which can be marked *e.g.* with a tetracycline label at the baseline of the experiment (Larmas & Kortelainen 1989). Also, the high-sucrose diet used in this experiment to induce caries is an independent factor disturbing dentin formation (Kortelainen & Larmas 1990, Tjäderhane *et al.* 1994, Huumonen *et al.* 2001). Therefore, to evaluate more accurately the effect of MMP inhibitors on dentin formation *in vivo*, an experiment without a high-sucrose diet but with the measurement of the dentin formed during the experiment would be useful.

6.5 MMPs in dentin (III, IV)

Even though mineralized dentin is not remodeled in the same manner as bone, it cannot be considered an inert tissue. Mineralized dentin contains various growth factors (reviewed by Goldberg & Smith 2004) and proteinases, including a latent collagenolytic enzyme (Dayan *et al.* 1983, Dumas *et al.* 1985) and gelatinolytic enzymes (Fukae *et al.* 1991), specifically MMP-2 (Martin-De Las Heras *et al.* 2000). Dentinal proteinases have been suggested to contribute to dentin caries progression (Dayan *et al.* 1983, Martin-De Las Heras *et al.* 2000, Tjäderhane *et al.* 2001a). They may also degrade the collagen fibrils involved in the adhesion of dental composite restorations (Pashley *et al.* 2004), leading to a failure of the restoration. However, dentinal proteinases may also have a defensive role during a caries attack, *e.g.* by releasing and activating growth factors needed in signaling for tissue repair in the dentin-pulp complex (Tjäderhane *et al.* 2001a).

MMPs are suggested to be involved in dentin formation (Tjäderhane *et al.* 1998a, Hall *et al.* 1999, Martin-De Las Heras *et al.* 2000, Palosaari *et al.* 2000), and at least some of these MMPs become embedded into mineralized dentin. The studies presented here demonstrate the presence of MMP-20 (III) and MMP-8 (IV) in mineralized human dentin. The previously published dentin protein extraction protocol (Martin-De Las Heras *et al.* 2000) was used to obtain the proteins of the non-mineralized dentin compartment in the remnants of the predentin layer and inside the dentinal tubules (G1 fraction), the mineral-bound proteins (E fractions), and the mineralized organic matrix (G2 fraction) (Fig. 2). Interestingly, dentinal MMPs, *i.e.* MMP-8, gelatinases (IV), and MMP-20 (III), showed different distribution profiles in the different compartments of dentin. The total amount of MMP-8 was highest in the non-mineralized dentin, whereas gelatinases showed a reversed but more moderate gradient, the relative gelatinolytic activity being greatest in the mineralized organic matrix (IV). On the other hand, MMP-20 was found in the mineral-bound proteins and the mineralized organic matrix, but not in the non-mineralized protein fraction (III). The differential distribution of MMPs in dentin

suggests the possibility of different roles in dentin. Their affinities to dentin may also differ. Since MMP-20 was absent from the G1 fraction in Western blotting but demonstrable in the corresponding predentin zone by IHC and soluble in dentinal fluid, it may have low affinity to dentin that is not mineralized. The reason for the discrepancy in the presence of gelatinases in mineral-bound protein (E) fractions between the results of study IV and the previous study (Martin-De Las Heras *et al.* 2000) remains unknown, since the extraction protocols were identical in these studies.

The presence of MMPs in the mineralized organic matrix and the mineral-bound protein fractions implies their embedding into dentin before and during mineralization. The mineral phase of dentin seems to protect at least gelatinases, since gelatinase activity could be demonstrated in autoclaved dentin (IV). Dentinal gelatinases and MMP-8 were mainly in an endogenously activated form (IV), as also observed in other studies (Martin-De Las Heras *et al.* 2000, Pashley *et al.* 2004). It is possible that they were activated during the extraction protocol, as observed previously with cartilage MMPs (Stanton and Fosang 2002), and alternative extraction protocols should be developed if the true nature of the activity of dentinal MMPs is to be elucidated. The presence of proMMPs in dentin (IV, Martin-De Las Heras *et al.* 2000) indicates that at least some dentinal MMPs exist in a zymogen form. However, the collagenolytic activity of untreated mineralized dentin powder (Pashley *et al.* 2004) indicates that part of dentin-bound MMPs exists truly in an endogenously active form.

Recent findings show that proMMPs exhibit proteolytic activity when bound to a specific ligand (Bannikov *et al.* 2002, Fedarko *et al.* 2004). Interestingly, particular small integrin-binding ligand N-linked glycoproteins (SIBLINGs) have been shown to bind and activate specific proMMPs without affecting the activity of the MMPs already in an active form (Fedarko *et al.* 2004). SIBLINGs are glycoproteins characteristically abundant in mineralized tissues, including dentin (Butler *et al.* 2003, Goldberg & Smith 2004). Of the SIBLING family members, bone sialoprotein binds and activates proMMP-2, osteopontin proMMP-3, and dentin matrix protein-1 proMMP-9 (Fedarko *et al.* 2004). This novel type of controlling MMP activity could be of great importance in dentin formation. Since the effect of the SIBLING proteins on specific MMP seems to involve a conformational change enabling proteolytic activity without cleavage of the propeptide (Fedarko *et al.* 2004), this type of activation would allow proteolytic activity with the propeptide still retained, and the proMMP protein could become embedded into dentin for future activation. In the future, protein extraction methods preventing MMP activation, effective at least in cartilage (Stanton and Fosang, 2002), could yield more specific information about the molecular forms of dentin-bound MMPs.

Recently, MMP-20 protein was detected in both presecretory and secretory odontoblasts, but not in dentin, with immunoelectron microscopy in rat incisors (Bourd-Boittin *et al.* 2004). This is consistent with our data on IHC staining of the dentin-pulp complex of fully developed human teeth, in which MMP-20 was not detected in mineralized dentin (III). However, we found MMP-20 in the mineralized organic matrix of dentin (G2 fraction) by a protein extraction protocol and Western blotting, and in carious teeth MMP-20 was seen in dilated dentinal tubules (III). Therefore, MMP-20 may be bound to normal mineralized organic matrix in a manner that hinders the interaction of the MMP-20-specific antibody used in these studies (III, Bourd-Boittin *et al.* 2004) and the targeted hinge region of the MMP-20 molecule.

MMP-2, -8, and -9 have been previously detected in soft carious dentin (Tjäderhane *et al.* 1998a). MMP-20 could be detected only occasionally by Western blotting (III), but IHC staining for MMP-20 showed immunoreactivity in some of the dilated dentinal tubules in the advancing front of caries lesions (III). The MMP-20 of carious dentin is currently the only MMP confirmed to be of the dentin-pulp complex origin, since whole saliva does not contain MMP-20 (unpublished observation). The other MMPs found in carious dentin, MMP-2, -8, and MMP-9 (Tjäderhane *et al.* 1998a), are present both in dentin (Martin-De Las Heras *et al.* 2000, IV) and in whole saliva (Ingman *et al.* 1994, Sorsa *et al.* 1990).

Carious dentin has been previously shown to exhibit increased collagenase activity, which has been suggested to be due to the activation of endogenous collagenase (Dayan *et al.* 1983). Indeed, dentinal collagenase can be "acid-activated" (Dung *et al.* 1995). Also, caries-like lesions can be induced on the root surface *in vitro* even in neutral pH without any exogenous proteases (Clarkson *et al.* 1986). Recently, no correlation between the extent of dentin collagenolysis *in situ* and the remaining dentinal gelatinolytic activity or activity against fluorogenic collagen-mimicking substrates could be observed (van Strijp *et al.* 2003). However, the findings of the study (van Strijp *et al.* 2003) do not exclude the possible importance of dentinal MMPs in collagenolysis or gelatinolysis in dentin organic matrix degradation during caries progression. If it is assumed that the gelatinase and collagenase levels of dentin are proportional to the weight (or volume) of dentin, the total MMP activity of the remaining "carious" dentin may not be substantially higher than the activity of native dentin, and the difference may not be statistically significant with small sample sizes. On the other hand, especially the collagenase activity of dentinal MMP-8 and MMP-2 may function as an initiator of tissue destruction, making the collagenous organic matrix also susceptible to non-specific proteases of host and microbial origin. In general, the exact significance of dentinal collagenase in dentin caries progression is very difficult to determine, since to date, the effects of this dentin-bound enzyme cannot be selectively excluded when using dentin as a substrate.

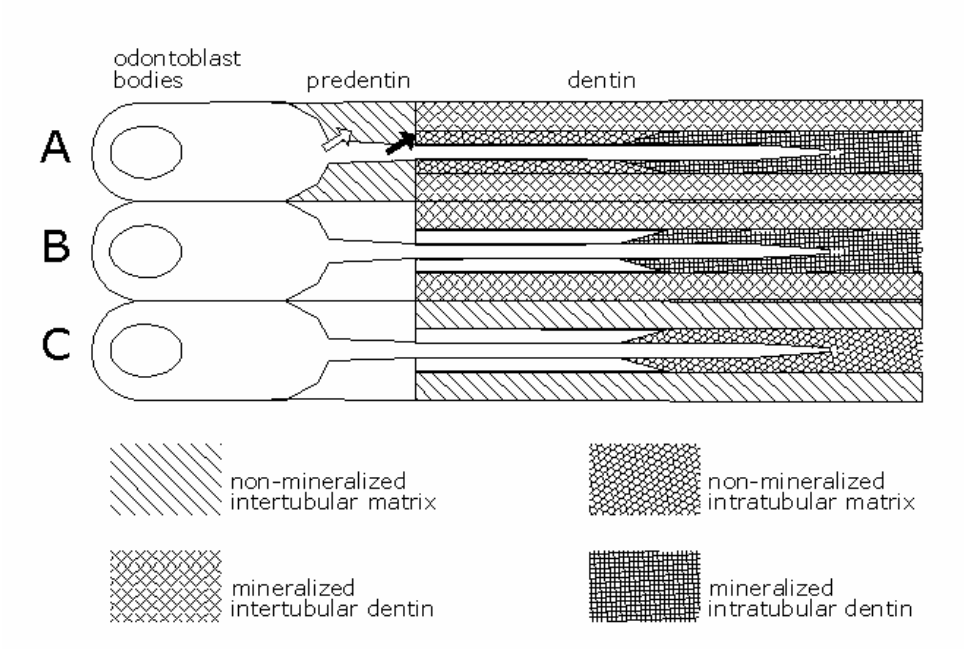

Fig. 2. A schematic representation of dentin structure and the dentin protein extraction protocol. Dentin is formed by odontoblasts, which have their processes inside the dentinal tubules. The organic matrix of intertubular dentin is secreted and reorganized in predentin and subsequently mineralized. Intratubular dentin forms inside the dentinal tubules, which also contain non-mineralized ECM and dentinal fluid. The first guanidine extraction (G1) removes the non-mineralized proteins of dentin (A → B). EDTA extractions (E) remove the minerals and related proteins (B → C), revealing the organic matrix of dentin, which is extracted with the second round of guanidine extraction (G2). Odontoblastic MMPs are found in predentin and non-mineralized intratubular ECM, in which they may contribute to ECM reorganization before mineralization (white arrow). Some MMPs remain in the organic matrix when it reaches the mineralization front, and/or they are secreted into the mineralization front, (black arrow), since some MMPs are found in mineralized dentin. MMPs, at least MMP-20, are also found in a soluble form in dentinal fluid.

7 Conclusions

The degradation of the collagenous organic matrix of dentin during caries progression is an enzymatic process. Some of the enzymes involved in this process may be of host origin, and specifically host MMPs were here hypothesized to take part in the breakdown of the dentin organic matrix. This hypothesis was supported by the reduced dentin caries progression in rats observed with the MMP inhibitors CMT-3 and zoledronate *in vivo*. However, the MMP inhibitors' other possible mechanisms of action need to be excluded before the significance of MMPs in dentin caries progression can be conclusively established. Also, the roles of MMPs originating from different sources, *i.e.* saliva, dentin, and the dentin-pulp complex cells, need to be determined in further studies. For example, even though CMT-3 inhibited salivary gelatinase activity *in vitro*, the evidence for the significance of salivary host MMPs in dentin caries progression remains inconclusive.

The studies on MMP expression in the dentin-pulp complex revealed high expression level of MMP-13 in pulp, which is rather exceptional in normal adult tissues. MMP-20 protein expression was demonstrated in odontoblasts and, to a lesser extent, in the pulp of fully developed teeth. The expression of MMP-13 and -20 in pulpal cells coincident with the abundance of tenascin-C, for example, may reflect the potential of these cells to differentiate into cells with mineralized matrix formation. Against the hypothesis of the study, pooled pulp samples failed to show any consistent and marked changes in the MMP or TIMP gene expression levels in response to caries by cDNA microarray. At the protein level, too, no major differences in levels of MMP-13 or -20 were observed between the pooled pulp and odontoblast samples of carious and sound teeth. However, the real-time quantitative PCR of individual pulp and odontoblast samples demonstrated a wide range of variation of relative MMP-13 mRNA expression, especially among pulp samples. These results emphasize the importance of combining approaches with pooled and individual samples by different methods when examining changes in MMP gene expression in the dentin-pulp complex cells in response to caries. Since the biological activity of MMPs is influenced by protein expression and, moreover, by the level of activation, future studies *e.g.* with protease-specific protein chips and protease activity chips (Lopez-Otin & Overall 2002) could provide valuable information on possible changes in the MMP activity of the dentin-pulp complex during caries progression.

Mineralized dentin constitutes a non-cellular source of MMPs in the dentin-pulp complex, as demonstrated by the presence of MMP-8 and -20 as well as the previously detected MMP-2 (Martin-De Las Heras *et al.* 2000) in human dentin. Dentinal MMPs are able to exhibit proteolytic activity against native and denatured type I collagen when released, and gelatinases, but not MMP-8 or MMP-20, seem to be protected by the mineral phase. The evaluation of the significance of dentinal MMPs in dentin caries progression provides a challenge for future studies, since it is difficult to selectively affect their activity.

In summary, several members of the MMP family are found in the soft and hard tissue compartments of the dentin-pulp complex. In addition to their presumed role in many physiological processes during the development and maintenance of the dentin-pulp complex, they may also contribute to the pathogenesis of dentin caries and the responses elicited by caries.

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