Faculdade de Odontologia de Piracicaba

FOP-UNICAMP

ANA PAULA DE SOUZA

EFEITO DE ÍONS METÁLICOS DIVALENTES SOBRE A ATIVIDADE DE METALOPROTEASES DA MATRIZ SECRETADAS POR CÉLULAS DO TECIDO GENGIVAL

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, para a obtenção do grau de Mestre em Biologia e Patologia Buco-Dental

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Orientador: Prof. Dr. Sérgio R. P. Line

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DEDICATÓRIA

A Deus, nosso Pai

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Agradeço e peço

Que ampare-nos

A fim de que o nosso sentimento

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E que possamos a cada dia

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SUMÁRIO

LISTA DE ABREVIATURAS e SIGLAS

MMP: Metaloprotease de Matriz

TIMP: Inibidor Tecidual de Metaloprotease

MMP-1: Colagenase (Fibroblastos)

MMP-2: Gelatinase A (Fibroblastos)

MMP-3: Estromelisina-1

MMP-8: Colagenase (Neutrófilos)

MMP-9: Gelatinase B (Neutrófilos)

MMP-10: Estromelisina-2

MMP-11: Estromelisina-3

kDa: k: quilo, Da=Dalton, (1 Da equivale a aproximadamente 1 unidade de massa atômica)

DMEM: *Dulbecco's Modified Eagle Medium*

PMN: Polimorfonuclear

SDS: Dodecil Sulfato de Sódio

EDTA: Ácido Etileno Diaminotetracético

PMSF: Fenilmetilsulfonil Fluoreto

NEM: N-Etilmaleimida

IL-1:Interleukin-1, Interleucina-1

EGF: *Epidermal Growth Factor,* Fator de Crescimento Epidermal

TNF: *Tumor Necrosis Factor,* Fator de Necrose Tumoral-α

PDGF: *Platelet-derived Growth Factor,* Fator de Crescimento Derivado de Plaquetas

TGF: Transforming Growth Factor, Fator do Crescimento Transformante

DMSO: Dimetil Sulfóxido

LMW: *Low Molecular Weigh,* Padrão de Baixa Massa Molecular

IgG: Imunoglobulina G

Con A: Concanavalina A

I_{50}: 50% de Inibição

Zn: Zinco

Cu: Cobre

Sn: Estanho

Hg: Mercúrio

Ca: Cálcio

Cd: Cádmio

Ni: Níquel

HCl: Ácido Clorídrico

$CaCl_2$: Cloreto de Cálcio

NaCl: Cloreto de Sódio

RESUMO & ABSTRACT

Matrix Metalloproteinases (MMPs) are a family of proteolytic enzymes that mediates the degradation of extracellular matrix. They are secreted as inactive proenzymes (zymogen), and they are thought to be activated in the tissue by cleavage of the propeptide. All members of this family have zinc and calcium binding to the active site. The MMPs take part in physiologic and pathologic events, as developmental of salivary glands and teeth and periodontal disease, respectively. Several divalent metal íons, as zinc and copper, are contained in dental materials, as dental amalgam, and the interaction between metal íons and the oral environment is a major subject in dental research. The aim of this work was to study the effect of several of divalent metals on the activity of MMPs secreted by gingival tissue cells. Gingival explants were cultured at 37° C for 24 h in DMEM and the secreted enzymes were characterized as MMP-2 and MMP-9 by immunoprecipitation. After, the effect of metals on the activity of the MMPs was tested using gelatin zymography and also against denatured collagen type I degradation *in vitro*. Divalente metals, as zinc and cupper, inhibited active and zimogen forms of MMP-2 and MMP-9.

Metaloproteases da matriz (MMPs) representam uma família de enzimas proteolíticas que participam da degradação da matriz extracelular. Estas enzimas são secretadas na forma de pró-enzimas inativas (zimógeno), sendo então ativadas na matriz extracelular por clivagem do pró-peptídeo. Todos os membros desta família apresentam um íon zinco e cálcio ligado ao seu sítio ativo . As MMPs estão envolvidas em fenômenos fisiológicos e patológicos, como o desenvolvimento das glândulas salivares e dos dentes, e na doença periodontal, respectivamente. Diversos íons metálicos divalentes, como zinco e cobre, estão contidos em materiais odontológicos, como o amálgama dental, e a interação entre metais e o meio ambiente oral têm sido assunto em pesquisa odontológica. Deste modo, o objetivo deste trabalho foi estudar o efeito de sais de diversos metais divalentes sobre a atividade de MMPs secretadas por células do tecido gengival. Fragmentos de tecido gengival foram incubados a 37° C por 24 h em DMEM e as enzimas secretadas foram caracterizadas por iminoprecipitação como MMP-2 e MMP-9. Posteriormente, o efeito destes metais sobre a atividade das MMPs foi testado utilizando zimografia de gelatina e também contra a degradação do colágeno tipo I desnaturado *in vitro*. Metais divalentes, como o zinco e cobre, mostraram potente efeito inibidor sobre a atividade da forma ativa e zimógeno da MMP-2 e MMP-9.

INTRODUÇÃO

Com o surgimento dos organismos multicelulares, houve a necessidade do desenvolvimento de uma matriz intercelular capaz de unir as células e servir de arcabouço. A matriz extracelular participa de muitos fenômenos celulares como diferenciação e crescimento. Esta matriz está constantemente sendo sintetizada e degradada. Membros das famílias das metaloproteases e dos ativadores de plasminogênio (serino proteases) são os principais reguladores da degradação da matriz extracelular (Birkedal-Hansen, 1993).

As metaloproteases compreendem uma família de enzimas que apresentam especificidade pelas macromoléculas da matriz extracelular. A família das metaloproteases é formada por vinte membros que exibem similaridades estruturais e funcionais (Tabela 1). As metaloproteases são secretadas na forma de zimógeno e como um complexo enzima-inibidor (Emonar e Grimaud, 1990; Stricklin *et al.*, 1983), sendo que sua ativação se dá em duas etapas. Inicialmente o zimógeno sofre clivagem proteolítica que resulta na remoção da porção amino-terminal. A clivagem pode ser feita por várias enzimas como a tripsina, plasmina, catepsina B e elastase. Numa segunda etapa, a enzima sofre autodigestão que resulta na sua forma ativada (Van Wart e Birkedal-Hansen, 1990). Acredita-se que a ativação é causada pela ruptura da ponte existente entre o aminoácido cisteína e o íon zinco, que

bloqueia o sítio ativo da molécula. Outra característica comum entre as metaloproteases é a dependência dos íons zinco e cálcio. A interação do zinco com dois resíduos de histidina presentes no domínio catalítico da molécula tem importância crucial para o funcionamento adequado das metaloproteases. Os dois átomos de cálcio conferem uma estabilidade para a estrutura terciária da proteína (Dioszegi *et al.*, 1995).

As metaloproteases desempenham papel importante em vários processos fisiológicos e patológicos, como na involução pós-parto (Weeks *et al.*, 1976; Souza *et al.*, 1989), na reabsorção óssea (Shapiro *et al.*, 1993 e Okada *et al.*, 1995), na inflamação através da migração leucocitária (Knauper *et al.*, 1993), na osteoartrite (Woessner, 1994 e Vijaykumar *et al.*, 1995), na doença periodontal (Birkedal-Hansen, 1993), no crescimento e expansão de tumores benignos (Autio-Hermainen *et al.*,1993) e na metástase (Declerck *et al.*, 1992).

As metaloproteases são divididas em 3 subclasses: colagenases intersticiais, gelatinases e estromelisinas. Esta classificação baseia-se na especificidade ao substrato. As colagenases intersticiais são as mais específicas. Foram as primeiras a serem descritas (Gross e Nagai, 1962) e, durante as últimas décadas, têm sido objeto de muitos estudos que versam sobre sua expressão e distribuição em processos normais e patológicos. Estas

enzimas são as únicas com capacidade de clivar as triplas hélices dos colágenos tipo I, II, III em condições fisiológicas, tornando estas moléculas susceptíveis à ação de outras enzimas (Souza *et al.*, 1993). Existem dois tipos de colagenases intersticiais que, apesar de exibirem grande semelhança estrutural e de especificidade, são codificadas por genes distintos. A do primeiro tipo (MMP-1), que exibe uma distribuição ambígua, é sintetizada por fibroblastos, queratinócitos, células endoteliais, macrófagos, osteoblastos, condrócitos e osteoclastos (Bikedal-Hansen, 1993). Quando analisada em eletroforese de gel de poliacrilamida a colagenase de fibroblastos aparece como várias bandas com massa molecular variando entre 57 e 52 kDa. Esta variação provavelmente se deve à proteólise parcial da molécula. A do segundo tipo é produzida exclusivamente por leucócitos polimorfonucleares (MMP-8). Apesar de possuir um núcleo protéico muito semelhante à colagenase de fibroblastos, a alta taxa de glicosilação faz com que esta molécula tenha massa molecular de 75 kDa. Ao contrário da colagenase de fibroblasto, que é rapidamente secretada após a sua síntese, a colagenase de polimorfonucleares é armazenada em grânulos intracelulares que só são liberados após a ativação das células (Hibbs e Bainton, 1989). Existem também as colagenases bacterianas que, clivam as cadeias alfa do colágeno nos inúmeros sítios ricos no

aminoácido glicina. Elas são muito utilizadas em laboratórios devido a sua grande especificidade a tripla hélice da molécula do colágeno.

As gelatinases são enzimas capazes de clivar a região helicoidal do colágeno tipo IV em dois fragmentos proporcionais a ¼ e ¾ do tamanho da molécula (Murphy *et al.*, 1989). Estas enzimas também são capazes de degradar colágeno tipo V e VII, elastina e gelatina (colágeno desnaturado). A gelatinase de 72 kDa (MMP-2) parece ser capaz de clivar também o colágeno tipo X (Welgus *et al.*, 1990), porém, não é ativa sobre o colágeno tipo I , II e III, laminina e proteoglicanos. A especificidade dessas enzimas sobre o colágeno tipo IV parece indicar que elas participam da remodelação e degradação da membrana basal. É também possível que a atividade gelatinolítica dessas enzimas esteja relacionada com a remoção de fragmentos de colágeno desnaturado que sofreram ação de outras metaloproteases (Reponen *et al.*, 1992 e Reponen *et al.*, 1994).

A gelatinase de 72 kDa é a metaloprotease mais abundante, sendo secretada por fibroblastos, queratinócitos, células endoteliais, macrófagos, osteoblastos e condrócitos, estando também presente no plasma sangüíneo (Birkedal-Hansen, 1993). A gelatinase de 92 kDa (MMP-9) é produzida por neutrófilos polimorfonucleares, queratinócitos e macrófagos, e,

ocasionalmente, por fibroblastos. Estudos de hibridização *in situ* têm demonstrado que as gelatinases de 92 kDa são fortemente expressas nos tecidos, embrionários de ratos (Reponen *et al.*, 1994).

O terceiro grupo de metaloproteases é formado pelas estromelisinas. As estromelisinas dos tipos 1 e 2 são capazes de digerir várias proteínas da matriz extracelular como a laminina, fibronectina, proteoglicano (núcleo protéico), colágeno tipo IV, V, VII e X, colágeno desnaturado (gelatina) e caseína (Chin *et al.*, 1985). A estromelisina 1 é expressa por várias células do estroma conjuntivo quando induzidas por fatores do crescimento e citocinas (IL-1, EGF, TNF-α e PDGF) (MacNaul *et al.*, 1990), e, quando analisada em gel de poliacrilamida, apresenta um massa molecular entre 60 e 53 kDa. Esta enzima parece não ser expressa por leucócitos ou queratinócitos. A estromelisina 2 é expressa em menor quantidade e, aparentemente, pelas mesmas células que expressam estromelisina-1. Apresenta um massa molecular semelhante à estromelisina-1 mas parece não responder a fatores de crescimento (Birkedal-Hansen, 1993).

Tabela 1. Resumo das principais enzimas que compõe a família das metaloproteases da matriz em humanos, com respectivas abreviações, massas moleculares e substratos.

ENZIMA	*ABREV.*	*MMP(kDa)*	*SUBSTRATO*
Colagenase de Fibroblasto	MMP-1	57/52	Colágeno I, ProMMP-2, ProMMP-9
Colagenase de Neutrófilo	MMP-8	85-64	Colágeno I
Colagenase-3	MMP-13	52-42	Colágeno I
Gelatinase A	MMP-2	72/66	ProMMP-9, Gelatina, Fibronectina, Elastina, Colágeno IV, V, VII e X
Gelatinase B	MMP-9	92/80	Gelatina, Fibronectina, Elastina, Colágeno IV, V, VII e X
Estromelisina-1	MMP-3	60/55	Fibronectina, Laminina, Elastina, Proteoglicanos, Caseína, Colágeno IV,V, IX, X, ProMMP-1,-7,-8,-9,-13
Estromelisina-2	MMP-10	60/55	Fibronectina, Laminina, Elastina, Proteoglicanos, Caseína, Colágeno IV, V, IX, X
MT1-MMP	MMP-14	66/54	ProMMP-2,-13, Colágeno I
MT2-MMP	MMP-15	72/60	----------
MT3-MMP	MMP-16	64/53	ProMMP-2
MT4-MMP	MMP-17	57/53	----------
Matrilisina	MMP-7	28/19	Fibronectina, Elastina, Colágeno IV
Metaloelastase	MMP-12	54/22	Elastina
Enamelisina	MMP-20	54/22	Matriz do Esmalte Dental

Fonte: Coletta *et al.*, 1996 / Woessner, 1998/ Johnson *et al.*, 1998

INIBIDORES DE METALOPROTEASES

A regulação da atividade proteolítica das metaloproteases ocorre em vários níveis, desde a regulação da transcrição até o controle da atrividade das MMPs na matriz extracelular através da ação de uma proteína inibidora de MMPs nos tecidos, a TIMP (inibidor tecidual de metaloprotease). Quatro membros da família TIMP: TIMP-1, -2, -3, -4, já foram caracterizados (Herron *et al.*, 1986). Estes inibidores estão distribuídos pelos tecidos e fluídos e são secretados por diversos tipos celulares, incluindo fibroblastos, neutrófilos polimorfonucleares, células endoteliais, condrócitos e células neoplásicas. O mecanismo de ação da TIMP é complexo, e envolve numerosos pontos de interação com as metaloproteases. O evento principal parece ser o resultado da ligação do inibidor ao domínio *hemopexin* da MMP, na região N-terminal da enzima (Murphy *et al.*, 1994). TIMP-1 parece ser mais efetivo na regulação das colagenases, enquanto que TIMP-2 é mais efetivo com o grupo das gelatinases (Howard *et al.*, 1991).

Há evidências da participação das MMPs na destruição periodontal e hoje é reconhecido que um desequilíbrio entre a atividade das MMPs e dos seus inibidores endógenos pode gerar um processo patológico de degradação da matriz extracelular durante a periodontite. Deste modo, existe grande

interesse no estudo de inibidores específicos sintéticos de MMPs, que possam ser utilizados como agentes terapêuticos no controle das MMPs.

Moléculas análogas às tetraciclinas, inibidoras de proteinases, modificadas quimicamente e sem propriedades antimicrobianas estão sendo estudadas em animais na prevenção da degradação do colágeno e da perda óssea durante processos de doença periodontal experimental. Múltiplos mecanismos de ação inibitória estão sendo propostos para estas substâncias, entre eles a capacidade que as tetraciclinas análogas teriam em prevenir a conversão de pró-MMPs em sua forma ativa (Ryan *et al.*, 1996; Souza *et al.*, 1998) ou devido à ação quelante destas drogas sobre metais que atuam como co-fatores para as MMPs.

Diclorometileno bifosfatado é uma droga que possui efeito inibitório sobre a reabsorção óssea e apresenta propriedades inibidoras de colagenase extracelular, um membro da família das metaloproteases. O mecanismo anticolagenolítico desta droga ainda não está claro, acredita-se que esta substância tenha a habilidade de quelar íons catiônicos (Teronen *et al.*, 1996; Souza *et al.*, 1998).

Substratos análogos de colagenase de fibroblasto e neutrófilos estão em desenvolvimento e em teste. Uma série de substratos análogos a base de

enxofre e sulfidrilas possuem propriedades inibidoras de colagenase, uma vez que estes substratos interagem com a enzima, como é o caso do grupo sulfidrila que é capaz de se ligar ao átomo de zinco do sítio ativo da MMP, formando um complexo que inibe a ação da enzima (Schwartz *et al.*, 1990; Souza *et al.*, 1998).

ARTIGOS

Inhibition of human gingival gelatinases (MMP-2 and MMP-9) by metal salts

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ABSTRACT

Objectives. The interaction between metal ions and the oral environment is a major subject matter in dental research. Matrix metalloproteinases (MMPs) have been implicated in several pathologic oral processes such as periodontal tissue destruction, root caries, tumor invasion and temporomandibular joint disorders. The aim of this work was to test the effect of Zn^{++}, Cu^{++}, Sn^{++}, and Hg^{++} ions, on the activity of the major gingival gelatinolytic MMPs.

Methods. Gingival explants were cultured overnight in DMEM and the activity of secreted enzymes was analyzed by gelatin zymography in buffers containing different metal ion concentrations. The major gelatinolytic proteinases present in the conditioned media were characterized as MMP-2 and MMP-9 by immunoprecipitation with specific antibodies. The eletrophoretic bands were scanned and the transmittance values were analyzed with the Sigmagel software (Sigma).

Results. $ZnSO_4$ was a strong inhibitor of MMP-2 (I_{50} = 15 μM) and MMP-9 (I_{50} = 40 μM), whereas $CuSO_4$, $HgSO_4$ and $SnCl_2$ showed less efficient inhibition potential.

Significance. Our findings show that the activity of oral tissue MMPs may be modulated by metal ions present in the oral environment. Therefore, the

accumulation of metals in connective tissue may interfere with the formation and resorption of the extracellular matrix components.

Keywords: gelatinases, MMP-2, MMP-9, metal, zinc, copper.

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of metal-dependent proteolytic enzymes that mediate the degradation of extracellular matrix and basement membranes. MMPs are secreted as inactive proenzymes (zymogens) and are thought to be activated in the tissue by cleavage of the propeptide. They have largely been described for vertebrate systems, but representatives of the family have also been found in plants, lower animals, and bacteria [1]. All members of this family have a zinc-and a calcium-binding catalytic domain, so that they depend on these ions for their activity. At least twenty MMPs have been characterized [1,2]. MMPs are widely distributed in the organism and are involved in physiologic as well as pathologic processes. These enzymes were shown to participate in the breakdown of extracellular matrix in disease processes as diverse as rheumatoid arthritis [3], atherosclerotic plaques [4] and cancer metastasis [5].

In the oral environment, MMPs take part in developmental events involving teeth and salivary glands and in collagen turnover [2,6,7], besides playing an important role in pathologic processes such as periodontal tissue destruction [8], oral lichen planus, dysplasia, squamous cell carcinoma [9], bone and cartilage degradation [10], and root caries [11,12,13]. Gingival fibroblasts, keratinocytes, resident macrophages and polymorphonuclear leukocytes (PMN) are capable of expressing MMP-1, MMP-2, MMP-3, MMP-8, and MMP-9 [14]. The importance of MMPs has raised interest in the development of inhibitors, and in recent years several molecules have been synthesized for potential therapeutic uses [15,16,17].

Several proteinases, including metalloproteinases, are known to be inhibited by zinc and other divalent metals [18,19,20,21,22]. Metals are extensively used in clinical dentistry, being present in restorative materials such as amalgam, metal alloys and zinc oxide-eugenol cements as well as in toothpastes and mouthrinses [23]. Soluble metal ions are constantly released from dental materials [24], and the effect of these ions on the oral environment has been a major subject matter in dental research [25]. In the present study we examined the effects of Zn, Cu, Sn and Hg ions on the activity of the major MMPs obtained from gingival explants of patients with periodontitis

MATERIALS AND METHODS

Collection and preparation of gingival metalloproteinases. Inflamed gingival tissue specimens were dissected from interproximal, lingual and buccal sites from 4 adult patients (3 females and 1 male aged 35 to 55 years) with periodontitis, requiring full-thickness flap surgery as part of their periodontal therapy. The study was carried out consistent with the principles of the Declaration of Helsinki and with the approval of the Human Research Ethics Committee of the University of Campinas, and all patients gave informed consent to participate.

Immediately following excision, the tissue specimens were pooled, washed in Dulbecco's Modified Eagle Medium (DMEM, GIBCO, New York, USA) containing 80 μg/mL Gentamicine at room temperature for 30 min. After washing, the specimens were incubated in DMEM containing 40 μg/mL Gentamicine at 37°C for 24 h. Following this step, gingival fragments were discarded and the cell culture conditioned medium (DMEM) containing the secreted MMPs was frozen at -70°C until analysis for enzyme activity.

Zymography. Proteolytic activity was examined on 10% polyacrylamide gels containing 0.05% gelatin. Conditioned medium was mixed with an equal volume of non-reducing sample buffer (2% SDS; 125 mM Tris-HCl, pH 6.8,

10% glycerol and 0.001% bromophenol blue) and then electrophoresed. After electrophoresis gels were washed twice in 2% Triton X-100 for 20 min at room temperature and then incubated at 37°C for 16 h in 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM $CaCl_2$ (Tris-$CaCl_2$). Following incubation, the gels were stained with 0.05% Coomassie Brilliant Blue G-250 (Bio Rad, Richmond, CA). Gelatinolytic activity was detected as unstained bands. The relative molecular masses of proteases were determined by the relation of log Mr to the relative mobility of Sigma SDS-PAGE LMW marker proteins.

Characterization of metalloproteinases by *enzyme inhibitors.* In order to examine the effect of inhibitors on enzyme activity, gelatin-containing gels were incubated in Tris-$CaCl_2$ buffer at 37°C for 16 h in the presence of the following inhibitors: 0.5 mM 1,10-phenanthroline (Sigma Chemical CO, St. Louis, MO, USA), 0.5 mM EDTA (Reagen, Brazil), 0.5 mM NEM (N-ethil-maleimide, Sigma) and 0,5 mM PMSF (Phenylmetylsulfonyl fluoride, Sigma).

Characterization of metalloproteinases by *immunoprecipitation.* Conditioned medium was subjected to an immunoprecipitation reaction with sheep anti-human MMP-2 IgG, and sheep anti-human MMP-9 IgG, (The Binding Site, Birmingham, England). The immunocomplexes were precipitated with protein A-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden) for 14

h at room temperature. After washing non-specific Sepharose-adsorbed material with TBS, the immunoprecipitated material was eluted with non-reducing sample buffer for 10 min at 70°C and assayed by gelatin zymography.

Inhibition of metalloproteinase activity by metal salts. In order to examine the effect of metal salts on the enzyme activity, conditioned medium containing MMPs was loaded on preparative gelatin-containing polyacrylamide gels. After electrophoresis the gels were cut in strips of 1 cm, and each strip was incubated at 37°C for 16 h in Tris-$CaCl_2$ buffer containing one of the following metal salts: $ZnSO_4$, $CuSO_4$, $SnCl_2$, $HgSO_4$. After adding the salts to the solution, pH was adjusted to 7.4. For some assays, after incubation with metal salts (37°C for 16 h in Tris-$CaCl_2$), the gels were extensively washed in 2 % Triton X-100, to remove soluble metal ions, and re-incubated in Tris-$CaCl_2$ solution for 16 h at 37°C. In order to quantify the relative inhibition of MMPs by $ZnSO_4$, $CuSO_4$, electrophoretic bands were scanned and the transmittance (the transmittance values of the zymogen and active form were added) was analyzed with the Sigmagel software (Sigma). Percent inhibition of enzyme activity was plotted against metal concentration. The inhibitory effect of the soluble metals was determined from the metal concentration (I_{50}) that inhibited 50% of the enzyme activity. The percentage

inhibition of $ZnSO_4$ and $CuSO_4$ was determined by comparing the activity of MMPs with control reactions, where these metals were not included (baseline value= 0% inhibition). Each assay was done in triplicate and was repeated at least two times.

RESULTS

Four major bands were detected in the zymographic assays (Fig 1), two sharper bands with an approximate molecular mass of 66 kDa and 72 kDa and two broader bands migrating between 80 kDa and 95 kDa. These enzymes were characterized as metalloproteinases since their activities were inhibited by phenantroline and EDTA (data not shown). PMSF (a serine-proteinase inhibitor) and NEM (a thiol-proteinase inhibitor) had no effect on enzyme activity (data not shown). The immunoprecipitation assays showed that the 66-72 kDa and 85-95 kDa enzymes corresponded to zymogens and active forms of MMP-2 and MMP-9, respectively (Fig. 1).

$ZnSO_4$ was a strong inhibitor of MMP-2 (I_{50}=15 μM) and MMP-9 (I_{50}= 40 μM) activities (Fig. 2). $CuSO_4$ had some inhibitory effect on MMP2 activity ((I_{50}= 1.5 mM), but did not have any effect on MMP-9 activity (Fig 3). The inhibitory effect of $ZnSO_4$ and $CuSO_4$ on MMP-2 activity was more

pronounced in the zymogen than in the active enzyme. The activities of MMP-2 and MMP-9 were fully inhibited by 3 mM $ZnSO_4$. This inhibition was only partially reversed after removal of soluble $ZnSO_4$ by extensive washing of the gels (Fig. 4).

The I_{50} concentration of $SnCl_2$ and $HgSO_4$ was not determined since these salts were poorly soluble in the Tris-$CaCl_2$ solution. Despite their low solubility, $SnCl_2$ and $HgSO_4$ showed some inhibitory effect on both MMP-2 and MMP-9 activities. However, inhibition was only noted when the precipitated salts were left in Tris-$CaCl_2$ solution, remaining in direct contact with the polyacrylamide gels (Fig. 5).

DISCUSSION

Zinc is extensively used in clinical dentistry. Besides being an important component of restorative materials, it is also used as an active component in toothpaste and mouthrinses. Clinical studies have shown that mouthrinses and dentrifices containing zinc salts can reduce plaque accumulation and calculus formation [26,27,28]. These effects are thought to be due to the anti-glycolytic and anti-ureolytic activity of zinc [29,30]. Additionally, zinc can reduce calculus formation by inhibiting hydroxyapatite crystal growth [31]. Using an

experimental gingivitis model, Saxton and Cummins [32] have also shown that zinc can effectively improve gingival health. These authors showed that dentrifices containing zinc citrate can reduce the development of gingival inflammation by 25%. The results presented in this paper show that besides reducing plaque growth, zinc can directly affect host responses by inhibiting MMP-2 and MMP-9 activity. MMP-2 and MMP-9, also known as gelatinases A and B respectively, are active in the degradation of denatured fibrillar collagens, elastase and several other components of the extracellular matrix [14,33,34]. There are several evidences indicating that MMP-2 and MMP-9 play an important role in tissue destruction during periodontal disease. Periodontitis patients have significantly higher levels of MMP-2 and MMP-9 than health subjects, and the amount of gelatinases decreases after periodontal treatment [33]. The activation of MMP-2 and MMP-9 was also shown to have a crucial role in the destruction of dentin by caries [13]. Additionally, these enzymes can potentate the degradation of extracellular matrix by activating collagenase-3 (MMP-13) and neutrophil collagenase [1]. Although the inhibitory effect of zinc can be partially reversed when metals are washed away, it has been shown that zinc is retained for at least 2 h in the mouth after tooth brushing with a 0,5% zinc-containing dentrifice [35]. The large amounts of zinc retained in plaque and on

the pellicle-covered tooth surface can prolong the exposure of oral MMPs to zinc. It is important to mention that normal adult human serum and saliva contain approximately 1.2 μg/mL (18 μM) and 0.2 μg/mL Zn (3 μM), respectively [35,36]. However, nearly all zinc present in the organic fraction of the body is bound to proteins. The I_{50} doses of $ZnSO_4$ reported here largely exceeds the amount of free zinc present in serum and saliva.

Zn, Cu, Hg, and Sn are metals commonly present in amalgam alloys. Large amounts of soluble metal ions are constantly released from amalgam [24,37,38]. The continuous salivary clearance would rapidly remove these metals from the mouth minimizing the interference with the MMPs present in saliva. However, in some cases such as amalgam tattoo and root-end filling, amalgam remains in direct contact with connective tissue for prolonged periods of time. The inhibition of MMP activity could have a local effect on the connective tissue around this material. There are many different formulations of amalgam with physical properties that may make them behave differently in regard to MMPs inhibition. Therefore, different formulations of amalgam may elicit different biological responses from connective tissue.

Several enzymes involved in the metabolism of extracellular matrix components are known to be inhibited by excess (>5 μM) metal ions. Cu and Cd

ions were shown to inhibit procollagen C- and N-proteinases, respectively [22]. Galactosylhydroxylysyl glucosyltransferase, an intracellular enzyme of collagen biosynthesis, is inhibited by excess of Zn^{2+}, Cu^{2+}, Cd^{2+}, Ni^{2+} and Ca^{2+} [18]. Excess Zn^{2+} can inhibit lysil and prolyl hydroxylation of collagen chains in various cell types. These findings, taken together with the results presented here, suggest that the accumulation of metals in connective tissue may interfere with the formation and resorption of the extracellular matrix components.

The mechanism of enzyme inactivation by metals is not completely understood. It is assumed that metal ions bind to specific sites causing conformational changes that inactivate the catalytic function of enzymes. Larsen and Auld [39] have shown that the mechanism of zinc inhibition of carboxypeptidase A, a zinc metalloproteinase, is due to the formation of zinc monohydroxide that bridges the catalytic zinc ion to a side chain in the active site of the enzyme. The non-competitive inhibition by other heavy metal ions is attributed to binding of the ion to a site distinct from the active site [20]. The fact that $ZnSO_4$ inhibition of MMP-2 and MMP-9 can only be partially reversed after removal of soluble salt suggests the existence of more than one zinc-binding site within these enzymes, where the low affinity interactions of zinc and MMPs can be disrupted by simply decreasing the amount of zinc.

Except for a few cytotoxicity models, pre-clinical testing of adverse effects of dental material suffers from the lack of suitable test methods [40,41]. The zymographic analysis using purified matrix proteinases or crude tissue extracts obtained from oral tissue can be used as an *in vitro* model of biocompatibility. This model differs from most *in vitro* biocompatibility tests which measure cytotoxicity by counting dead cells or by using metabolic parameters [42]. Zymographic analysis can be used as an additional parameter, and in combination with these traditional systems can provide more accurate and comprehensive information on the biocompatibility of materials and chemicals used in dentistry.

ACKNOWLEDGMENTS

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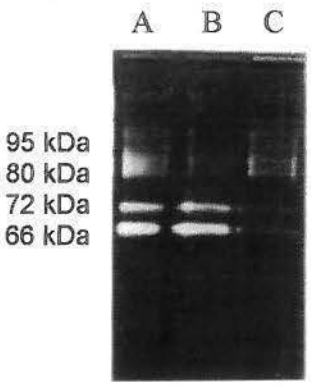


Fig. 1. Gelatin zymography of immunoprecipitated MMPs. (A) Control lane containing conditioned media; (B) MMP-2 immunoprecipitated material; (C) MMP-9 immunoprecipitated material.

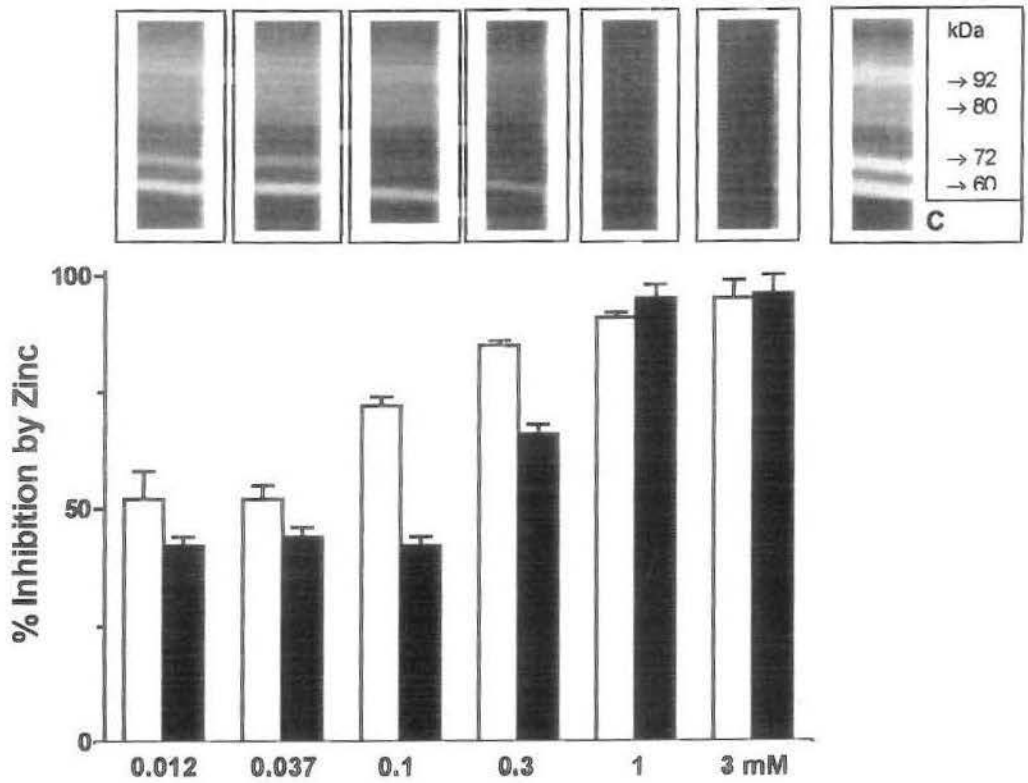
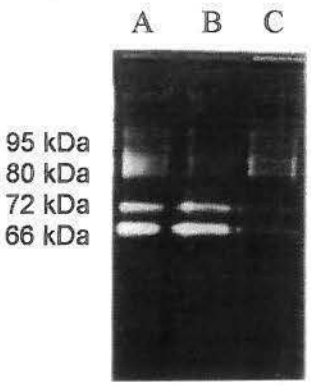


Fig. 2. Dose response of inhibition of zymogen and active forms of gingival MMP-2 (□) and MMP-9 (■) by $ZnSO_4$ ± SD. The corresponding gelatin zymography is shown above bars. The control zymography, where $ZnSO_4$ was not included, is shown in (C).



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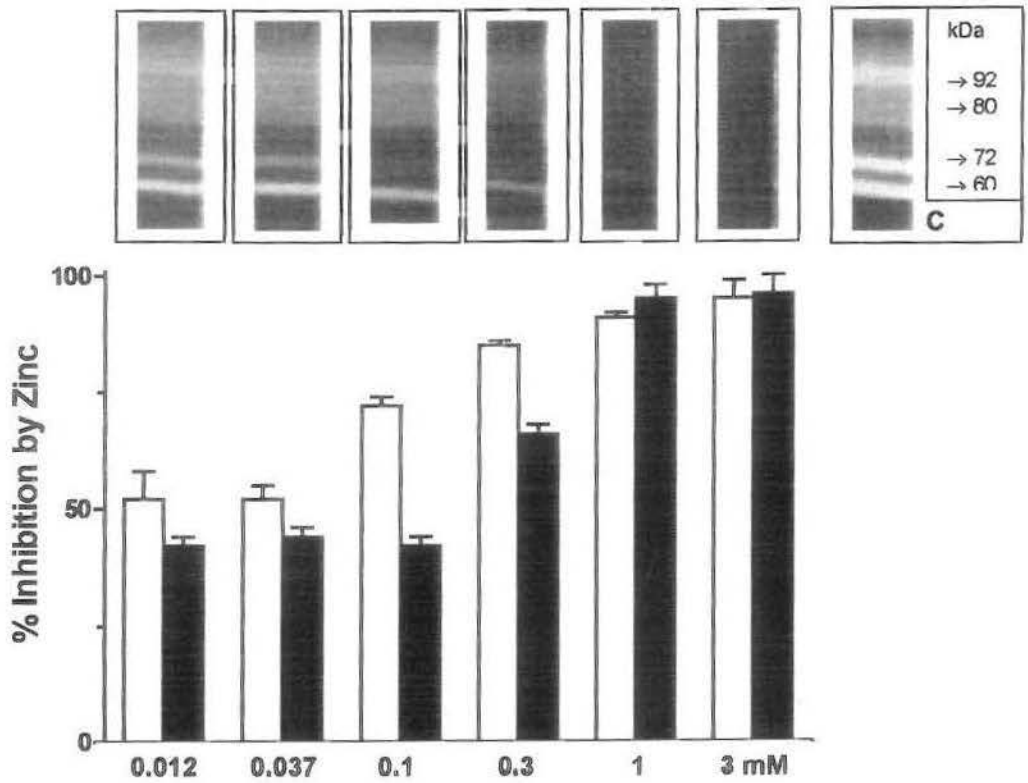

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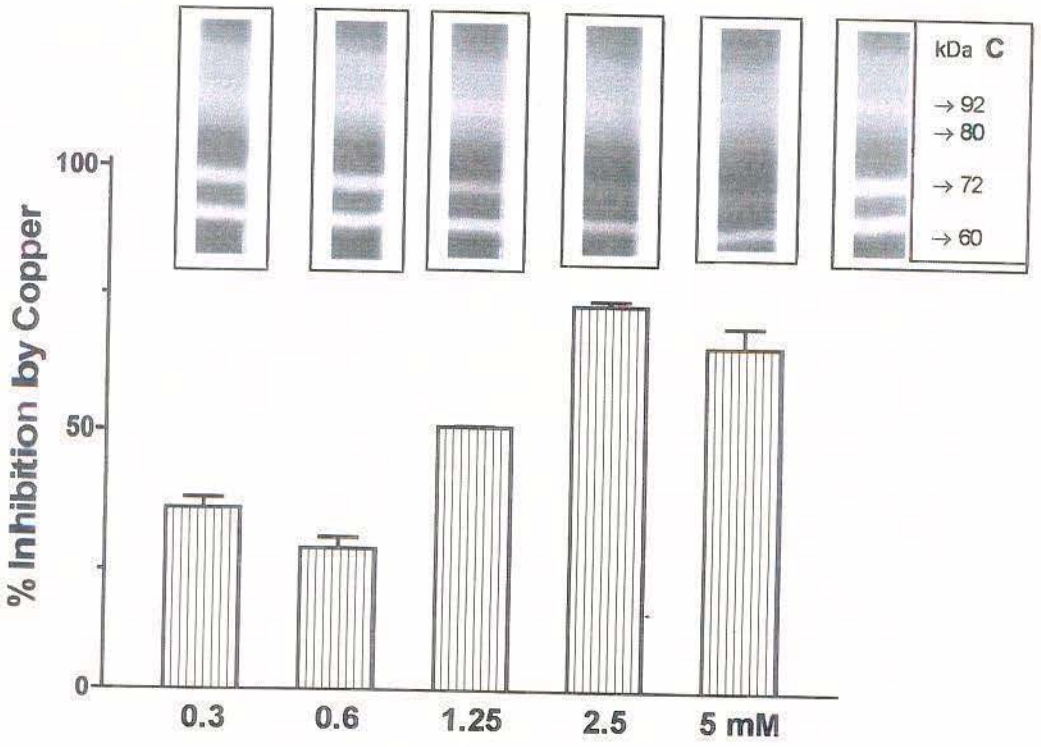

Fig. 3. Dose response of inhibition of zymogen and active forms of gingival MMP-2 by $CuSO_4$ ±SD. The corresponding gelatin zymography is shown above bars. The control zymography, where $CuSO_4$ was not included, is shown in (C). Note that $CuSO_4$ did not have any effect on MMP-9 activity.

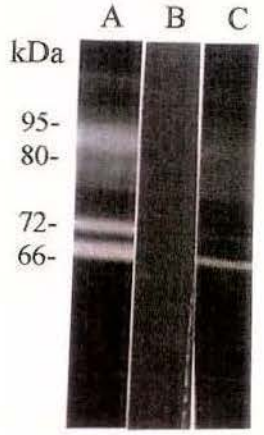

Fig. 4. Gelatin zymography showing the reversal of the inhibitory effect of $ZnSO_4$. Conditioned medium was loaded on a preparative. (A) Control lane incubated with Tris-$CaCl_2$ buffer only. (B) Inhibition with 3 mM $ZnSO_4$. (C) Reversal of the inhibitory effect of 3 mM $ZnSO_4$ after removal of soluble zinc ion by extensive washing of the gel and reincubation in Tris-$CaCl_2$ buffer. Note that MMP-2 and MMP-9 activities were partially restored.

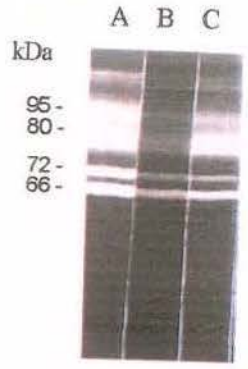

Fig. 5. Gelatin zymography showing the inhibition of $HgSO_4$ and $SnCl_2$. Gel strips were incubated with a mixture containing 40 mL of Tris-$CaCl_2$ buffer and 5 mM of $SnCl_2$ and $HgSO_4$, respectively. These salts were fairly insoluble forming a precipitate which remained in direct contact with gel strips during the incubation period. (A) Control lane incubated with $CaCl_2$ buffer only. (B) Inhibition by $HgSO_4$. (C) Inhibition by $SnCl_2$.

Inhibition of Human Gelatinases by Metals Released from Dental Amalgam

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Running title: Amalgam inhibition of human gelatinases

Key words: metal, dental amalgam, gelatinases, MMP, zinc

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Abstract. The interaction between metal ions and the oral environment is a major subject matter in dental research. Matrix metalloproteinases (MMPs) have been implicated in pathologic oral processes such as periodontal tissue destruction, root caries, tumor invasion and temporomandibular joint disorders. The aim of this study was to test the effect of metal ions released from dental amalgam on the major gingival gelatinolytic MMPs. Gingival human explants were cultured overnight in DMEM and the activity of secreted enzymes was analyzed by gelatin zymography in buffers conditioned with dispersed phase or concentional phase dental amalgams. The major enzymes present in conditioned media were characterized as MMP-2 and MMP-9 by immunoprecipitation. The proteolytic activities of MMP-2 and MMP-9 were strongly inhibited by dispersed phase amalgams conditioned buffers. Inhibition of MMP-2 and MMP-9 activities was partly reversed by the addition of 1,10 phenanthroline, a divalent metal chelator, to the amalgam conditioned buffers. Dental amalgam conditioned buffer also inhibited the degradation of denatured type I collagen by purified MMP-2, on liquid phase assays. These findings suggest that the activity of oral tissue MMPs may be modulated by metal ions released from dental amalgam.

Introduction

Dental amalgam, an alloy formed by mercury with another metal or metals, is one of the oldest materials used in restorative dentistry, and still is one of the most commonly employed. Several metal ions are constantly released from amalgam by electrochemical reactions (Brune, 1981; Jensen, 1983; Johansson and Dérand, 1983). Part of these ions are released into the saliva and oral fluids, but products of biodegradation of dental restorations are also transported into the pulp, tooth structure, and soft tissues (Kurosaki and Fusayama, 1973; Lindh and Tveit, 1980). Amalgam used in root end fillings or amalgam debris occasionally embedded in the oral mucosa in the form of amalgam tattoo can release metal ions directly into the connective tissue. Ions can also be transported through the tooth into the circulation system of body (Eley and Garret, 1983). The magnitude of the effects of these metals on the immediate environment and the consequences are a major subject matter in dental research (Marek, 1992).

Metalloproteinases (MMPs), an important family of metal-dependent endopeptidases, are responsible for the degradation of extracellular matrix components. At least twenty MMPs have been characterized and all members

of this family have a zinc and a calcium binding catalytic domain, so that they depend on these ions for their activity. These enzymes are secreted by various human cells as inactive proenzyme (zymogens) and are thought to be activated in the tissue by proteolytic cleavage of the propeptide (Birkedal-Hansen, 1993). In the extracellular matrix, specific tissue inhibitors of MMPs (TIMPs) control the enzyme activity by forming a stoichiometric enzyme-inhibitor complex with the activated enzyme.

In the oral environment, MMPs take part in development events involving teeth, salivary glands and collagen turnover (Barlett *et al.*, 1996; Golub *et al.*, 1995; Ingman *et al.*, 1996), besides playing an important role in pathologic processes such as periodontal tissue destruction (van der Zee, 1996), and root caries (Dayan *et al.*,1983; Kawasaki and Feathertone, 1997; Tjaderhane, 1998).

We have recently shown that MMP-2 is strongly inhibited by excess of Zn^{++}, whereas a Cu^{++}, Sn^{++} and Hg^{++} exhibited a less efficient inhibition potential (Souza *et al.*, 1999). Since zinc is the major corrosion product released from conventional and dispersed phase amalgams (Marek, 1992), we have investigated the effects of corrosion products from zinc-containing and zinc-free dental amalgam on the activity of human gelatinases.

Materials and Methods

Collection and preparation of gingival metalloproteinases

Human gingival tissue specimens were dissected from adult patients (3 females and 1 male aged 35 to 55 years) with periodontitis, requiring full-thickness flap surgery as part of their periodontal therapy. The study was carried out consistent with the principles of the Declaration of Helsinki and with the approval of the Human Research Ethics Committee of the University of Campinas, and all patients gave informed consent to participate. Immediately following excision, the tissue specimens were washed in Dulbecco`s Modified Eagle Medium (DMEM, GIBCO, New York, USA) containing 80 mg/mL gentamicin at room temperature for 30 min. After washing, the specimens were incubated in DMEM containing 40 mg/mL gentamicin at 37°C for 24 h. Following centrifugation, aliquots of supernatant were frozen at -70°C until analysis for enzyme activity.

MMP-2 Purification

Gelatinases were partially purified from other MMPs present in the supernatant by affinity chromatography using gelatin-Sepharose 4B (Pharmacia, Piscataway, NJ) and eluted using 0-10% dimethyl sulfoxide

(DMSO) gradient. Gelatin-Sepharose-bound enzymes were dialyzed in 50 mmol/L Tris-HCl, pH 7.4, containing 5 mmol/L $CaCl_2$. A second chromatography step using Concanavalin A (Con A)-Sepharose (Sutton, 1995) which was required to separe MMP-2 and MMP-9. MMP-2 did not bind to concanavalin A. Due to the small amount of protein obtained, MMP-2 concentration was estimated by comparing the intensity of silver stained MMP-2 bands with the intensity of Sigma SDS-PAGE marker proteins. Purified MMP-2 was stored in liquid nitrogen.

Preparation of Dental Amalgam Conditioned Buffer and Determination of Zinc Concentration

One dental amalgam capsule of a zinc-containing high-copper alloy amalgam (Dispersalloy®, DENTSPLY/Caulk, Milford U.S.A.), was placed in 40 mL 50 mmol/L Tris-HCl, pH 7.4 containing 5 mmol/L $CaCl_2$ (Tris-$CaCl_2$) at 37°C. The buffer (amalgam conditioned Tris-$CaCl_2$ buffer) was changed daily, and the fractions were stored at –20°C until used. Zinc released in the daily fractions was determined by flame atomic absorption spectroscopy with Varian model SpectrAA 50 spectrometer using the standard flame and detection conditions, and manual sample injection. A standard curve was constructed in the range 3-30 μmol/L. Tris-$CaCl_2$ buffer was used as the blank.

The zinc concentration of each sample was calculated as the mean of three repetitions.

Zymography

Proteolytic activity was examined on 10% polyacrylamide gels containing 0.05% gelatin. The supernatant was mixed with an equal volume of non-reducing sample buffer (2% SDS; 125 mmol/L Tris-HCl, pH 6.8, 10% glycerol and 0.001% bromophenol blue) and then electrophoresed. After electrophoresis gels were washed twice in 2% Triton X-100 for 20 min at room temperature and then incubated at 37°C for 16 h in Tris-$CaCl_2$ buffer. Following incubation, the gels were stained with 0.05% Coomassie Brilliant Blue G-250 (Bio Rad, Richmond, CA). Gelatinolytic activity was detected as unstained bands. The relative molecular masses of proteases were determined by the relation of log Mr to the relative mobility of Sigma SDS-PAGE LMW marker proteins.

Characterization of metalloproteinases by immunoprecipitation.

The supernatant was subjected to an immunoprecipitation reaction with sheep anti-human MMP-2 IgG, and MMP-9 IgG (The Binding Site, Birmingham, England). The immunocomplexes were precipitated with protein A-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden) for 14 h at room

temperature. After washing non-specific Sepharose-adsorbed material with 50 mmol/L Tris pH 7.4, 200 mmol/L NaCl, the immunoprecipitated material was eluted with non-reducing sample buffer for 10 min at 70^0C and assayed by gelatin zymography

Inhibition of metalloproteinase activity using zymography

In order to examine the effect of metals released amalgams on enzyme activity, medium containing MMPs was loaded on preparative gelatin-containing polyacrylamide gels. After electrophoresis the gels were cut in strips of approximately 1 cm (this assures that each strip has the same concentration of enzymes), and each strip was incubated at 37°C for 24 h in amalgam conditioned buffer. Following incubation the gel strips were stained with Coomassie blue as described in zymography. We have used two zinc-containing amalgams: Dispersalloy® and Standalloy® SF (Degussa SA, Brazil); and a zinc-free amalgam: Standalloy® F (Degussa SA, Brazil). Standalloy® F and Standalloy® SF have the same formulation, except that Standalloy® F contains no zinc.

In order to demonstrate that the inhibition of MMP-2 activity was caused by divalent metals released from amalgams we have added different amounts

of 1,10-phenanthroline, a divalent metal chelator, to the amalgam conditioned Tris-$CaCl_2$ buffer.

Assay for MMP Inhibition

Since in zymography enzyme (gel) and inhibitor (solution) are not in the same reaction-phase, we have used another gelatinase activity assay providing the same soluble reaction phase for MMPs, substrates, and inhibitors. Crude extract (MMPs conditioned DMEM) or purified MMP-2 (0.75 ug) were activated by incubation at 37°C for 30 min in 0.5 mmol/L *p*-aminophenylmercuric acetate (APMA). The effect of amalgam conditioned solution (Dispersalloy®) on MMPs activity was tested as follows. Activated MMPs were incubated with amalgam conditioned buffer at 37°C for 30 min. The final volume was completed to 135 μL with 50 mmol/L Tris-HCl pH 7.4 containing 5 mmol/L $CaCl_2$ and 10 mmol/L PMSF. 15 μL of gelatin at 3 mg/mL were added, and the assays were further incubated at 37°C for 24 h. Gelatin was prepared by treating rat type I collagen at 100°C for 30 min. 1,10-phenanthroline at 3 mmol/L was used as a control for inhibition of MMPs activity. After incubation, electrophoresis sample buffer was added (75 μL), and the samples were heated at 70°C for 5 min and then resolved on 10% SDS-polyacrylamide gels. Proteins were stained with Coomassie Blue G-250. In

order to quantify the relative inhibition of MMPs by zinc, electrophoretic bands were scanned and the absorbance was analyzed using the Sigmagel software (Sigma).

Since zinc has been shown to be the major metal released from Dispersalloy® (Brune, 1981), and to have the highest potential for MMP-2 and MMP-9 inhibition, when comparing to other metals released from dental amalgam (Souza *et al*, 1999), the percent inhibition of MMP-2 activity was plotted against zinc concentration.

The inhibition of MMPs activity were determined by comparing the effect of amalgam conditioned buffers with control reactions containing only Tris-$CaCl_2$ buffer.

Results

Four major bands were detected in the zymographic assays (Fig 1), two sharper bands with an approximate molecular mass of 66 kDa and 72 kDa and two broader bands migrating between 80 kDa and 95 kDa. These enzymes were characterized as metalloproteinases since their activities were inhibited by phenantroline and EDTA (data not shown). PMSF (a serine-proteinase

inhibitor) and NEM (a thiol-proteinase inhibitor) had no effect on enzyme activity (data not shown). The immunoprecipitation assays showed that the 66-72 kDa and 85-95 kDa enzymes corresponded to zymogens and active forms of MMP-2 and MMP-9, respectively (Fig. 1).

Figure 2 shows that MMP-2 and MMP-9 activities were strongly inhibited by buffer solutions conditioned by zinc-containing amalgams, whereas only mild inhibition was observed by buffer conditioned by zinc-free amalgam. Figure 3 shows the inhibitory activity of the daily fractions of Dispersalloy® conditioned buffer. Some inhibition of MMP-2 activity was observed up to day 7, while MMP-9 inhibition was only noted until day 4.

In order to show that the inhibition of MMPs activities was caused by metals released from amalgam we have added 1,10 phenanthroline, a divalent metal chelator, to Dispersalloy® conditioned Tris-$CaCl_2$ buffer. Figure 4 shows that inhibition of MMP-2 and MMP-9 activities could be reversed by 1,10 phenanthroline. The inhibition observed with concentrations higher than 0.5 mmol/L 1,10 phenanthroline was likely due to zinc chelation from the active site of MMPs.

The inhibitory potential of metals released from Dispersalloy® conditioned Tris-$CaCl_2$ solution was also tested against MMPs conditioned

DMEM or purified MMP-2, on a soluble reaction phase. Figure 5 shows that MMP-2 (0.75 μg) degradation of denatured type I collagen was inhibited by metals released from amalgam. Dispersalloy® conditioned buffer also inhibited the degradation of denatured type I collagen (not shown). However, some degradation was noted in the reaction incubated with 1,10 phenanthroline, indicating that other enzymes were present in this crude preparation.

Discussion

We have shown that divalent metal ions released from dental amalgam can effectively inhibit MMP-2 and MMP-9 proteolytic activities. These enzymes, also known as gelatinase A and gelatinase B, respectively, are active in the degradation of denatured fibrillar collagens, elastin and several other components of the extracellular matrix (Creemers *et al.*, 1998; Birkedal-Hansen *et al.*, 1993). There are several evidences indicanting that MMP-2 and MMP-9 play an important role in tissue destruction during periodontal disease (Creemers *et al.*, 1998; Ingman *et al.*, 1994; Makela *et al.*, 1994). The activation of MMP-2 and MMP-9 was also shown to have a crucial role in the destruction of dentin by

caries (Tjaderhane, *et al.*, 1998; Kawasaki and Featherstone, 1997) and in the formation and remodeling of extracellular matrix during wound healing (Woessner, 1990). Additionally, these enzymes can potentiate the degradation of extracellular matrix by activating collagenase-3 (MMP-13) and neutrophyl collagenase (MMP-8) (Murphy and Knauper, 1997).

Although the toxic effects of dental amalgam have been largely imparted to the release of mercury, studies have reported that zinc is also highly cytotoxic when tested *in vitro* (Marek, 1992; Molberg and Johansson, 1991; Mueller and Edahl, 1984). The continuous salivary clearance would rapidly remove these metals from the mouth minimizing the interference with the MMPs present in saliva. However, in some cases such as amalgam tattoo and root-end filling, amalgam remains in direct contact with connective tissue for prolonged periods of time. The inhibition of MMP activity could have a local effect on the connective tissue around this material and this may alter the formation and resorption of the extracellular matrix components, interfering with the healing or remodelling of the tissues around these materials.

Dental pulp reaction in response to soluble molecules from restorative materials is inversely related to the dentin thickness, and is proportional to the diffusion rate through dentin (Hanks et al, 1994). There is

some evidence that metal ions released from amalgam can reach the pulp (Kurosaki and Fusayama 1973, Marshal et al 1980, McTigue et al 1984). Since MMPs are known to participate in the mineralization of pre-dentin matrix, the inhibition of these enzymes could interfere with the calcification of reparative dentin.

There are several evidences indicating that zinc is responsible for most of the inhibition observed in our results. Data from the literature show that the amount of zinc released from Dispersalloy® is at least 50X higher than that of copper or mercury (Brune 1981). Souza *et al.*, (in press) have shown that MMP-2 is strongly inhibited by excess of Zn^{++}, whereas a Cu^{++}, Sn^{++} and Hg^{++} produced a less efficient inhibition. Finally, the inhibition caused by zinc containing amalgams (Dispersalloy® and Standalloy® SF) was stronger than the inhibition observed with zinc-free amalgam (Standalloy® F). The rate of zinc release from Dyspersalloy® was very high, as has been shown for zinc-containing amalgams (Brune 1981, Jensen 1983, Johanson and Dérand 1983). Most zinc was released during the first 24 h, but the conditioned solution collected on day 7 still had some inhibitory effect on MMP-2 activity. Although *in vitro* conditions can not be extrapolated for *in vivo* situations, high

concentrations of soluble metal ions are likely to be found in the local environment around dental amalgam for long periods.

Several enzymes involved in the metabolism of extracellular matrix components were also shown to be inhibited by excess (>5 μM) metal ions. Cu and Cd ions were shown to inhibit procollagen C- and N-proteinases, respectively (Hojima *et al.*, 1994). Galactosylhydroxylysyl glucosyltransferase, an intracellular enzyme of collagen biosynthesis, is inhibited by excess of Zn^{2+}, Cu^{2+}, Cd^{2+}, Ni^{2+} and Ca^{2+} (Myllyla *et al.*, 1979). Excess Zn^{2+} can inhibit lysil and prolyl hydroxylation of collagen chains in various cell types. The mechanism of enzyme inactivation by metals is not completely understood. It is believed that metal ions interact with amino acid residues causing conformational changes that inactivate the catalytic function of enzymes. Larsen and Auld (1991) have shown that the mechanism of zinc inhibition of carboxypeptidase A, a zinc metalloproteinase, is due to the formation of zinc monohydroxide that bridges the catalytic zinc ion to a side chain in the active site of the enzyme. The non-competitive inhibition by other heavy metal ions is attributed to binding of the ion to a site distinct from the active site (Mallya and Van Wart, 1988).

Except for a few cytotoxicity models, pre-clinical testing of adverse effects of dental material suffers from the lack of suitable test methods

(Schmalz, 1996,1998). Divalent metals are extensive used in clinical dentistry. In addition to amalgam, metals are important components of cementing media, cavity liners, temporary filling of teeth, root canal filling materials and tooth pastes. The zymographic analysis using purified matrix proteinases or crude tissue extracts obtained from oral tissue can be used as an *in vitro* model of biocompatibility for any metal-containing material. Since dental materials normally contain other biologically active components; such as eugenol, detergents and formaldehyde; the addition of 1,10 phenanthroline can be used to assess the participation of metals in the inhibition of MMPs. Furthermore, this model differs from most *in vitro* biocompatibility tests which measure cytotoxicity by counting dead cells or by using metabolic parameters (Hanks *et al.*, 1996). Zymographic analysis can be used as an additional parameter, and in combination with these traditional systems can provide more accurate and comprehensive information on the biocompatibility of materials and chemicals used in dentistry.

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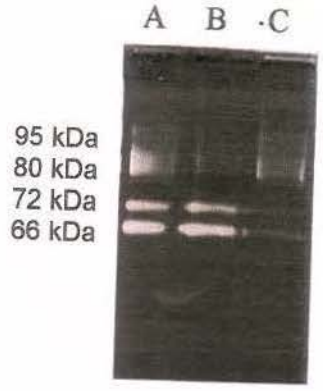

Fig. 1. Gelatin zymography of immunoprecipitated MMPs. (A) Control lane containing conditioned media; (B) MMP-2 immunoprecipitated material; (C) MMP-9 immunoprecipitated material.

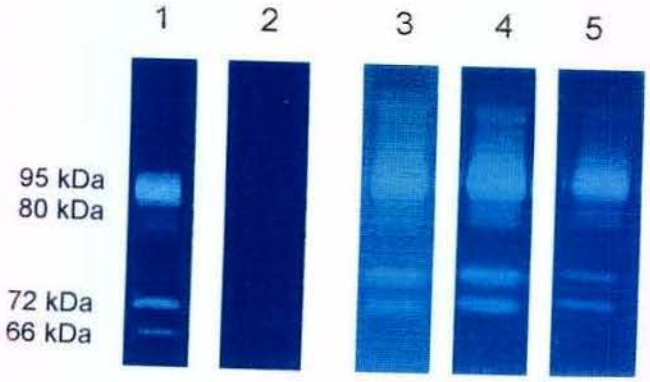

Fig. 2. Gelatin zymography showing the effect of two different dental amalgams on MMPs activities. Gel strips were incubated in 40 mL of Tris-$CaCl_2$ buffer conditioned by zinc-containing or zinc-free dental amalgam. (1) Control lane incubated with Tris-$CaCl_2$ buffer only. (2) Dispersalloy® conditioned Tris-$CaCl_2$ buffer. (3) Control lane incubated with Tris-$CaCl_2$ buffer only. (4) Standalloy® F conditioned Tris-$CaCl_2$ buffer. (5) Standalloy® SF conditioned Tris-$CaCl_2$ buffer. Note that zinc-free amalgam (Standalloy® F) had little effect on MMP-2 and MMP-9 activities.

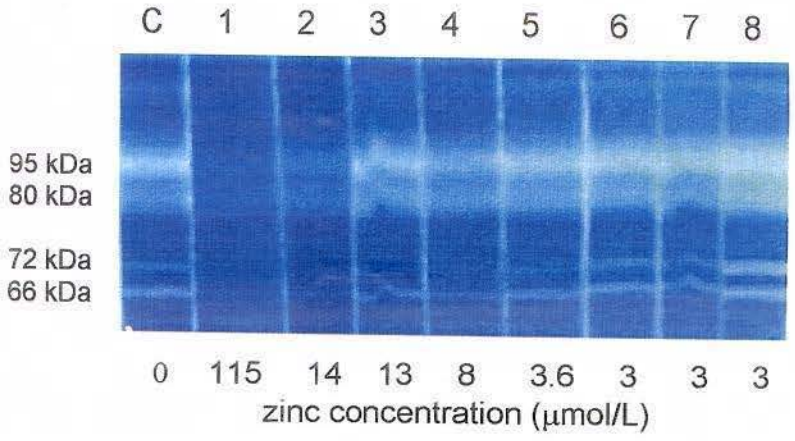

Fig. 3. Gelatin zymography showing the inhibition of MMPs activities by the daily fractions of Dispersalloy® conditioned Tris-$CaCl_2$ buffer until 8 days of incubation. The concentration of zinc released into the buffers measures are shown bellow each lane.

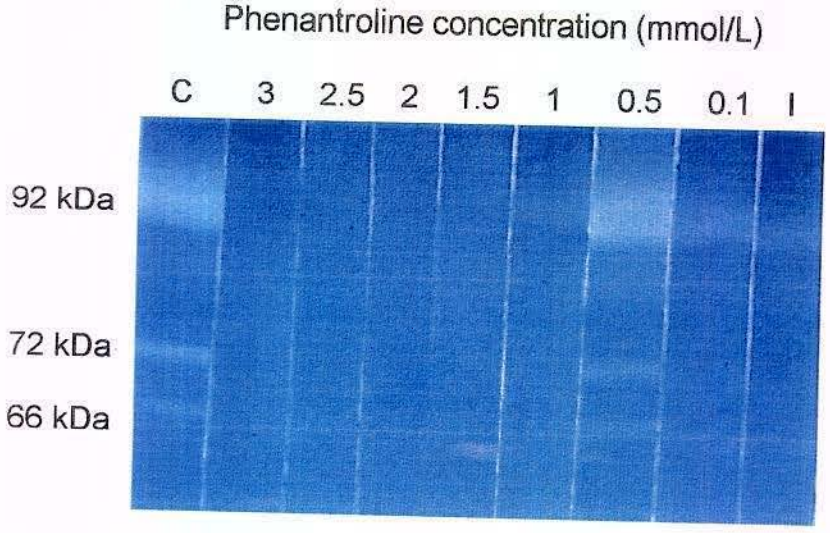

Fig. 4. Gelatin zymography showing the activity restored of MMPs by 1,10-phenantroline. Note 0.5 mmol/L 1,10-phenantroline removed excess zinc from buffer but not the active site zinc of enzymes. The inhibitions observed with concentrations higher than 0.5 mmol/L 1,10-phenantroline were probably due to chelation of zinc ions from the active site of MMPs. (C) Control lane containing conditioned media. (I) Inhibition of MMPs by metals released of dental amalgam.

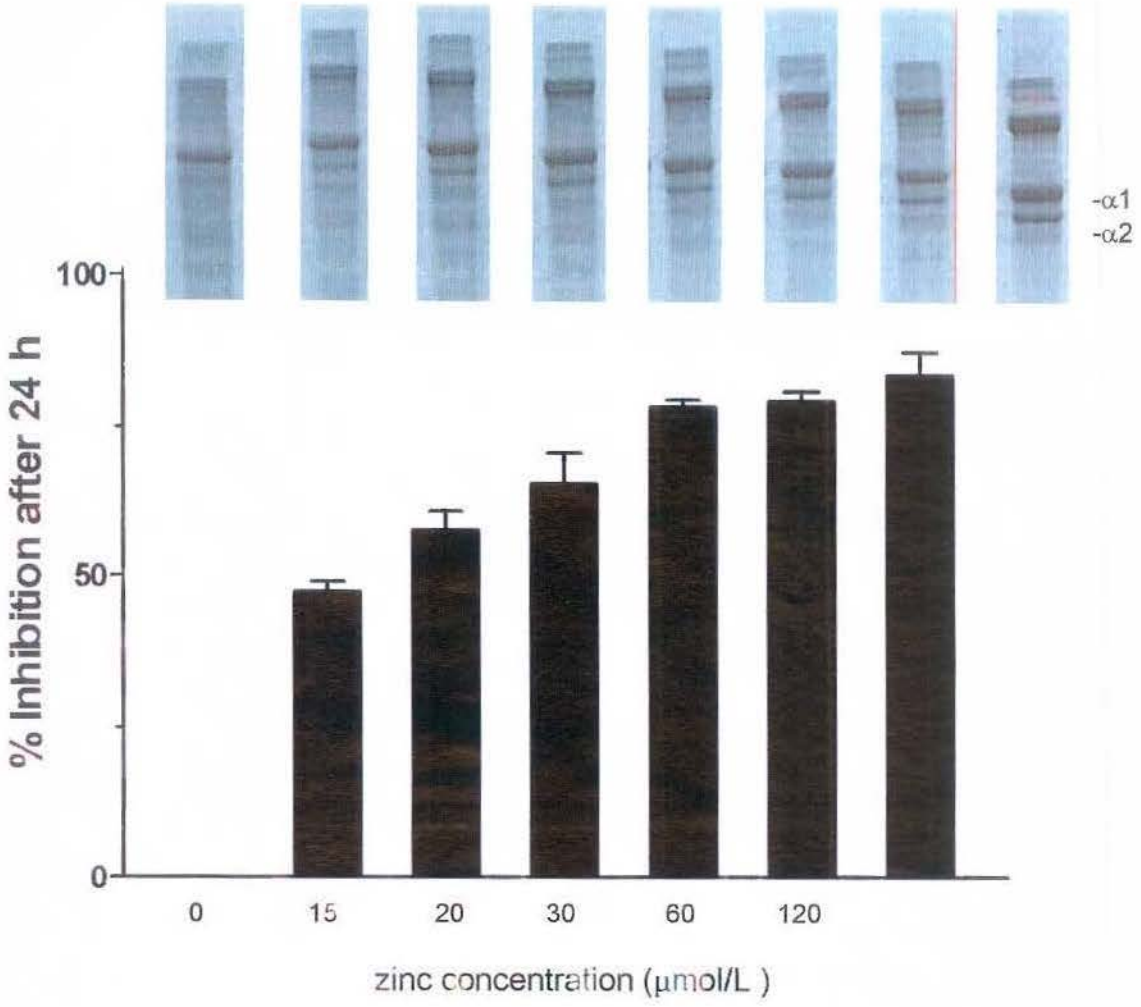

Fig. 5. Dose response of inhibition of purified MMP-2 by metals released from dental amalgam ± SD. The corresponding gels of denatured type I collagen degradation are shown above and the zinc concentration in buffer are shown bellow bars. The inhibition control, where 3 mmol/L 1,10-phenantroline were added, is shown in (P). The control, where there was only denatured type I collagen, is shown in (C).

CONCLUSÕES

1. Sais de metais divalentes como sulfato de zinco ($ZnSO_4$) ou sulfato de cobre ($CuSO_4$) demonstraram alta capacidade de inibição da atividade da MMP-2 e da MMP-9 *in vitro*, utilizando como método a zimografia.

2. Amálgama dental contendo zinco (Dispersalloy®) demonstrou liberar nas primeiras 24 h após seu preparo quantidade suficiente de metais para provocar inibição da atividade da MMP-2 e da MMP-9, utilizando como método a zimografia, e inibição da MMP-2 isolada, utilizando método de degradação do colágeno desnaturado *in vitro* pela enzima.

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ANEXOS

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PERSPECTIVAS PARA O USO DE INIBIDORES ENZIMÁTICOS NA TERAPIA PERIODONTAL

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SINOPSE

A doença periodontal é um processo iniciado pelo acúmulo de placa bacteriana sobre a superfície dental, que gera inflamação nos tecidos vizinhos afetados, a gengiva e o ligamento periodontal. As primeiras fases da inflamação acarretam acúmulo de leucócitos polimorfonucleares (PMNs), principalmente neutrófilos, células inflamatórias que produzem enzimas responsáveis pela degradação de macromoléculas da matriz extracelular. Estas enzimas, chamadas de metaloproteases (MMPs), desempenham papel fundamental na patogênese da periodontite, iniciando a quebra da molécula do colágeno e tornando esta suscetível à ação de outras enzimas presentes na matriz extracelular. Esta revisão da literatura discute as substâncias em pesquisa que têm por finalidade inibir a atuação destas MMPs durante a doença periodontal.

UNITERMOS: Periodontite, Metaloproteases, Inibidores de MMPs

ABSTRACT

Periodontal disease is iniciated by the deposition of bacterial plaque on the dental surface, resulting in inflammation of the surrounding affected tissues, the gingiva and the periodontal ligament. Inflammation process initial steps involve the accumulation of polimorphonuclear neutrophil leukocytes, inflammatory cells that produce enzymes responsible for degradation of collagen and other extracellular macromolecules. These enzymes, called matrix metalloproteases (MMPs), play a fundamental role in periodontitis pathogenesis, since they have the unique ability to cleave native type I collagen to be further degraded by other proteolytic enzymes with wider substrate specificities. This literature review discusses substances that are currently under research and may inhibit MMPs in periodontal disease.

KEY WORDS: Periodontal disease, Metalloproteinases, Inhibitor MMPs.

INTRODUÇÃO

Com a diminuição da prevalência da doença cárie, os problemas que atingem o periodonto passaram a ser a principal causa de perdas dentais nas sociedades ocidentais. A gengivite representa o início da inflamação gengival, que se não controlada, evolui para a doença periodontal destrutiva.

A periodontite é uma doença humana crônica que se instala no periodonto, um órgão com características anatômicas e fisiológicas peculiares e exposto a um microambiente único, em que a ameaça microbiana pode ser muito variável. A compreensão da patogênese da doença periodontal ainda é limitada, o que faz com que o tratamento seja predominantemente voltado para o controle da causa mais aceita (a placa microbiana).

Com o acúmulo de conhecimentos a respeito da biologia do periodonto, surgiram novas técnicas para a terapia periodontal. A regeneração tecidual guiada surgiu ao se perceber que as células epiteliais têm uma velocidade migratória maior do que as células do conjuntivo[13]. O condicionamento ácido da superfície radicular foi sugerido depois de se perceber a atividade citotóxica da camada superficial da dentina depois da instrumentação periodontal [22]. O uso de tetraciclinas e seus análogos foi preconizado quando se comprovou a inibição da degradação de colágeno promovida por estas drogas [7, 17].

Os recentes avanços nas pesquisas sobre inibidores de enzimas que degradam colágeno abrem perspectivas para novas terapias periodontais. O objetivo desta revisão é discutir o importante papel da inibição de metaloproteases na prevenção da degradação periodontal e os diversos inibidores destas enzimas.

REVISÃO DA LITERATURA

Periodonto Normal

O periodonto é o único órgão formado por dois tecidos conjuntivos frouxos, a gengiva e o ligamento periodontal, e dois tecidos calcificados, o cemento e o osso alveolar. Como todos os demais tecidos conjuntivos, a matriz extracelular do periodonto é composta por proteínas colágenas, não-colágenas e proteoglicanas. Embora a proporção dos diferentes tipos de colágenos varie nos diferentes tecidos do periodonto, o colágeno tipo I é o predominante em todas as estruturas normais [12]. Os estímulos locais como as forças oclusais e a placa bacteriana contribuem para que "turn-over" do colágeno no periodonto seja o mais alto de todo o organismo. Isto explica por que o escorbuto, doença em que há deficiência na síntese de colágeno, manifesta-se primeiramente no periodonto, gerando o afrouxamento dos dentes.

Na gengiva, as fibras de colágeno estão arranjadas como feixes densos ou como fibras finas e curtas dispersas na forma de uma rede, sendo os tipos I e III os mais abundantes. No ligamento periodontal predomina colágeno tipo I, o qual também constitui o componente fibroso do osso alveolar. Este colágeno é o componente principal das fibras de Sharpey, as quais contêm também os colágenos tipo III, V, VI e XII. A matriz orgânica do cemento é composta predominantemente de colágenos tipo I e III. Os colágenos também são os principais constituintes da matriz do osso alveolar.

Alterações bioquímicas do periodonto decorrentes da doença periodontal

Por causa de sua estrutura anatômica, o periodonto é severamente afetado por alterações decorrentes da inflamação. O periodonto é a localização primária de várias doenças, sendo algumas sistêmicas e algumas induzidas por drogas. A gengivite é uma das doenças humanas mais comuns. Nesta lesão, os tecidos conjuntivos gengivais são destruídos depois de três a quatro dias de acúmulo de placa bacteriana, e está associada à migração de PMNs para a região do epitélio juncional e sulco gengival. A destruição do colágeno começa nas proximidades dos vasos sangüíneos. Aproximadamente 70% do colágeno dos focos de inflamação é perdido como conseqüência da liberação de enzimas armazenadas nos PMNs. A gengivite pode progredir para a periodontite, pode durar meses ou até décadas, ou regredir após a instituição do controle de placa adequado [23]. A periodontite crônica leva a uma extensa perda do tecido conjuntivo gengival, ligamento periodontal, osso alveolar e, algumas vezes, superfície radicular. As células destrutivas mais importantes são macrófagos e PMNs. Grandes quantidades de PMNs podem causar destruições recorrentes em ciclos de doença. O nível de colágeno é reduzido e pode ocorrer fibrose do tecido gengival em focos de inflamação nos casos de doença de progressão lenta. Com o avanço da doença, a destruição pode atingir o osso alveolar e as superfícies radiculares [14].

Alterações quantitativas e qualitativas ocorrem nos colágenos gengivais na doença periodontal. Na gengiva, o colágeno se torna mais solúvel, indicando síntese de novo colágeno ou síntese deficiente de colágeno [1].

Aceita-se hoje a existência de duas vias para a degradação do colágeno do periodonto. A primeira via de degradação é através de fagocitose por fibroblastos do periodonto, e esta é a via implicada na remodelação do tecido normal. A outra via é aquela em que a degradação do colágeno acontece pela ação das enzimas pertencentes à família das metaloproteases, e está associada a inflamação. Ambas as vias são mediadas pela ação das citocinas, sendo o TGF-b (fator transformador do crescimento) o responsável pela indução da degradação por fagocitose, enquanto que a IL-1a (interleucina 1a) é a responsável pela ativação das metaloproteases teciduais e pela secreção de metaloproteases por células inflamatórias [18]. Alguns autores têm conseguido demonstrar que a doença periodontal parece exibir surtos de destruição tecidual e outros períodos de estabilidade da condição. Esta teoria relaciona as perdas de suporte com os períodos de ativação das colagenases intersticiais e os períodos de estabilidade com as fases em que estas enzimas estão sendo secretadas depois da exaustão dos períodos de atividade de doença periodontal [21].

Ação das Metaloproteases na degradação dos colágenos

As metaloproteases da matriz (MMPs) são uma família de enzimas proteolíticas que regulam a degradação de macromoléculas da matriz extracelular, entre elas o colágeno. O nome "metalo" se deve ao fato destas enzimas dependerem de metais como cofatores. Estas enzimas são secretadas na forma latente e tornam-se ativas na matriz extracelular conforme estímulos locais [1].

A família desta enzimas é dividida em quatro grupos. O primeiro grupo é formado por enzimas

secretadas por fibroblastos e PMNs (a MMP-1 e MMP-8), colagenases que degradam colágeno tipo I, II, III, VII, VIII, X e gelatina, que é a forma denaturada do colágeno. O segundo grupo inclui enzimas com peso molecular de 72 kDA (MMP-2) e 92 kDA (MMP-9), que degradam colágeno tipo IV e gelatina. As estromalisinas formam o terceiro grupo e degradam as proteínas centrais das glicosaminoglicanas. O último grupo é formado por outras enzimas que se supõe serem metaloproteases mas ainda não são bem compreendidas (Tabela 1).

queratinócitos, os macrófagos e os PMNs. As metaloproteases mais importantes durante a doença periodontal são a MMP-1 e a MMP-8. A MMP-1 é secretada por fibroblastos, macrófagos, células endoteliais, células epiteliais e osteoblastos, sendo também conhecida por colagenase intersticial ou de fibroblastos. A MMP-1 é secretada na forma inativa e ativada no tecido por outras enzimas, como a MMP-3 e a plasmina. Já a MMP-8 é secretada na forma ativa por PMNs. Os PMNs são capazes de liberar uma

destas enzimas na patogênese da doença periodontal [2].

Bactérias patogênicas associadas à periodontite também são capazes de produzir enzimas destruidoras de colágeno, mas o papel destas enzimas bacterianas na destruição do tecido gengival é desprezível em comparação com a destruição causada pelas enzimas teciduais [11].

A ação destas enzimas parece estar restrita às fases ativas de destruição durante a periodontite e deve contribuir para o sucesso ou

Tabela 1: Classificação das MMPs

Enzimas	Abrev.	PM(kDa)	Substrato
Colagenase de fibroblastos	MMP-1	57/52	Colágeno tipo I, II, III, VII, VIII, X e gelatina
Colagenase de PMN	MMP-8	75	Colágeno tipo I,II,III, VII, VIII, X e gelatina
Estromalisina-1	MMP-3	60/55	Fibronectina, Laminina, Elastina, Colágeno tipo IV, V, IX, X, Proteoglicanos e Caseína
Estromalisina-2	MMP-10	60/55	Fibronectina, Laminina, Elastina, Colágeno tipo IV, V, IX, X, Proteoglicanos e Caseína
Estromalisina-3	MMP-11	____	
Gelatinase-A	MMP-2	72	Gelatina, Fibronectina, Elastina, Colágeno tipo IV, V, VII e X
Gelatinase-B	MMP-9	92	Gelatina, Elastina, Colágeno tipo IV e V
Matrilisina	MMP-7	28	Fibronectina, Laminina, Elastina, Proteoglicanos, Colágeno tipo IV
Metaloelastase de Macrófagos	MMP-12	55	Elastina

Classificação baseada em Coletta "et alii" [3].

Muitas das doenças inflamatórias crônicas que destroem colágeno e matriz extracelular estão envolvidas com a ação descontrolada das MMPs. Entre estas doenças podemos destacar a periodontite, a artrite reumatóide e processos de metástase, neste último exemplo estas enzimas favorecem a infiltração de células tumorais através da degeneração do tecido conjuntivo.

A maioria das células do periodonto produz MMPs em situação normal e durante a inflamação, entre elas os fibroblastos, os

grande quantidade de MMP-8 acumulada em seus grânulos durante a inflamação, promovendo grande destruição tecidual. A grande importância da MMP-1 e da MMP-8 é o fato de serem as precursoras na degradação do colágeno tipo I, razão por que são também conhecidas como colagenases. A ação destas enzimas torna a molécula do colágeno passível de degradação por outras enzimas. Apesar de se ter reconhecido sua importância na destruição periodontal, não foi esclarecida ainda a contribuição relativa de cada uma

fracasso clínico de procedimentos como a regeneração tecidual guiada (RTG). GROSSO et alii [5] mostraram em estudo *in vitro* que células removidas de membranas de politetraflúormetileno (PTFM), utilizadas em RTG, exibiam diferentes níveis de atividade de MMP-1 e MMP-8. Nos casos clínicos onde ocorreu o fracasso da técnica observou-se a expressão extremamente alta destas enzimas.

Muitos casos de doença periodontal são acompanhados também pelo aparecimento de cárie

radicular. Naturalmente o processo carioso decorre de conhecidos fatores relacionados ao hospedeiro, como a dieta e a microflora. Entretanto, a cárie radicular relacionada à doença periodontal está associada com dois outros fatores de risco: a perda do cemento, com conseqüente exposição da dentina, e a ação de MMPs, secretadas por bactérias da bolsa e pelo próprio tecido gengival. Sendo o cemento uma estrutura com menor quantidade de matéria inorgânica, e maior quantidade de matriz protéica, e sendo esta composta basicamente por colágeno, as colagenases atuam destruindo esta parte orgânica e contribuindo para o desenvolvimento da lesão cariosa na porção radicular [10]. No caso da dentina exposta, a ação das MMPs é ainda mais devastadora do que sobre o cemento, uma vez que a maior parte da estrutura orgânica da dentina é composta por colágenos dos tipos I e III.

DISCUSSÃO

Inibidores de MMPs

A regulação proteolítica das metaloproteases ocorre a nível extracelular, através da ação de uma proteína inibidora específica de tecidos, a TIMP (inibidor tecidual de metaloprotease). Dois membros da família TIMP: TIMP-1 e TIMP-2, já foram caracterizados [6]. Estes inibidores estão distribuídos pelos tecidos e fluídos e são secretados por diversos tipos celulares, incluindo fibroblastos, PMNs, células endoteliais, condrócitos e células neoplásicas. O mecanismo de ação da TIMP é complexo e envolve numerosos pontos de interação com as MMPs. Atualmente se reconhece que um desequilíbrio entre a atividade das MMPs e a ação dos inibidores endógenos pode gerar uma degradação patológica do colágeno da matriz extracelular durante a periodontite [15]. Isto gera o interesse no estudo de inibidores específicos sintéticos de MMPs que possam ser utilizados clinicamente como agentes terapêuticos na periodontite.

As tetraciclinas são o primeiro grupo de drogas usadas como agente auxiliar no tratamento de doenças do periodonto. Estas drogas possuem efeito antibacteriano de amplo espectro. Além desta ação, as tetraciclinas têm a capacidade de inibir colagenase (MMP-8, colagenase de PMNs), propriedade que não esta ligada a sua ação antibacteriana, uma vez que, tetraciclinas análogas, modificadas quimicamente e sem ação bacteriana, também apresentam capacidade de inibir as colagenases. O mecanismo de ação desta droga sobre a enzima é discutível, mas pode estar relacionado à capacidade em prevenir a conversão da forma inativa para a forma ativa da enzima [12]. As tetraciclinas não atuam sobre a MMP-1 (colagenase de fibroblasto) do fluído do sulco gengival, indicando que estas enzimas são mais resistentes a ação da droga. As colagenases de pacientes adultos com problemas periodontais são mais suscetíveis à tetraciclina enquanto que a forma de periodontite juvenil é relativamente mais resistente [17,7].

Diclorometileno bifosfatado (Clodronate) tem sido utilizado para diminuir a degradação do colágeno em vários tipo de tumores associados com metástase óssea e para diminuir as reações inflamatórias de doenças como as artrites. Na odontologia, estas drogas apresentam capacidade de inibir a progressão da destruição óssea durante a periodontite. Resultados de pesquisas indicam que estas drogas inibem a ação da MMP-1 quelando cátions. Esta droga possui baixo nível de toxicidade e ação de longa duração, índices satisfatórios que tornam o Clodronate viável para o tratamento de doenças que destroem osso e matriz extracelular [19]. Esta droga tem substituído a tetraciclina em algumas pesquisas que a utilizam como terapia química auxiliar de implante dental e de regeneração tecidual guiada [20].

Em relação a implantes, é interessante um estudo de ISHIGURO et alii [8], que sugere que MMPs e TIMPs encontrados ao redor de implantes seriam os responsáveis pelos insucessos de implantes de prótese de quadril em condições assépticas. É possível que o uso de inibidores de MMPs aumente o índice de sucessos em implantes, inclusive os implantes bucais.

Algumas pesquisas apontam para a utilização de substâncias que tenham capacidade de competir com o colágeno pelas enzimas. São substratos análogos, à base de sulfidrilas e enxofre, que se ligam ao sítio ativo da enzima evitando que esta degrade o colágeno, o substrato natural. Desta forma, as MMPs seriam inibidas através da interação entre enzima-inibidor [16].

O fluoreto estanhoso, utilizado em alguns dentifrícios, foi associado a uma diminuição da inflamação gengival e do sangramento da gengiva. Acreditava-se que este fato era decorrente do controle inibitório na formação da placa bacteriana que o dentifrício provocaria. Entretanto, alguns estudos comprovam que este benefício não corresponde ao decréscimo no escore de placa bacteriana [9]. Uma hipótese possível é a de que o estanho inibiria a atuação das colagenases de PMNs no tecido gengival, acarretando uma diminuição da inflamação e do sangramento. Além do fluoreto estanhoso, outros metais são utilizados em alguns dentifrícios e podem ser importantes para a diminuição do sangramento gengival associada com o uso destes dentifrícios [4]. Entre estes metais, citamos o sal metálico cloreto de zinco, visto que em estudos preliminares de nosso laboratório se demonstrou que o zinco tem a capacidade de inibir colagenase intersticial de fibroblastos gengivais *in vitro*. A figura 1 mostra um zimograma de poliacrilamida a 10% que revela a inibição da atividade enzimática pelo zinco.

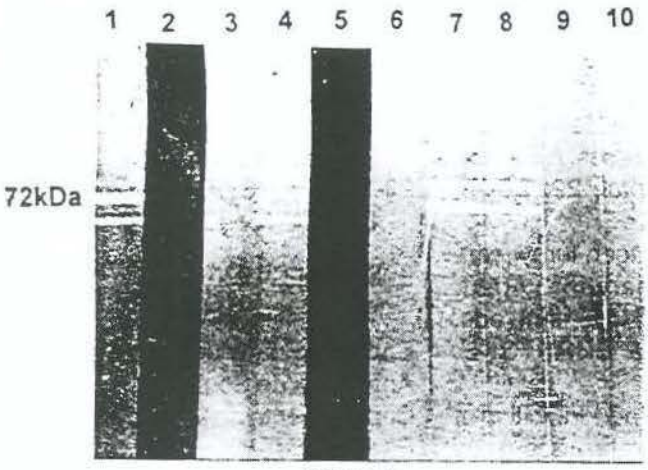

Figura 1

Figura 1 - Zimografia em gel de poliacrilamida contendo gelatina. Depois da separação, o gel foi dividido em fitas e encubado com diferentes íons divalentes em tampão TRIS-HCl/$CaCl_2$ por 16 horas. As bandas brancas visíveis são áreas em que as enzimas degradaram a gelatina do gel. As bandas correspondem à MMP-2, o que se sabe pelo peso molecular desta enzima.
1-Controle (somente o tampão TRIS-HCl/$CaCl_2$).
2-Tampão + Ferro reduzido (10 mM).
3-Tampão + Ferrocianeto de Potássio (10 mM).
4-Tampão + Cloreto de Magnésio (10 mM).
5-Tampão + Cloreto Férrico (10 mM).
6-Tampão + Cloreto de Zinco (10 mM).
7-Tampão + Sulfato de Potássio (10 mM).
8-Tampão + Sulfato de Magnésio (10 mM).
9-Tampão + Sulfato de Cobre (10 mM).
10- Tampão + Sulfato de Zinco (10 mM).

CONCLUSÃO

O conhecimento da biologia do periodonto possibilita a descoberta de novos métodos terapêuticos que possam estabilizar a destruição tecidual durante a periodontite. As enzimas da família das metaloprotreases sem dúvida atuam no avanço da perda de estruturas de suporte, através da destruição do colágeno no meio extracelular. Conhecer o funcionamento de inibidores de MMPs pode permitir o implemento de seu uso como meio auxiliar na terapia periodontal.

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FACULDADE DE ODONTOLOGIA DE PIRACICABA

UNIVERSIDADE ESTADUAL DE CAMPINAS

PARECER DO CEP — FOP/UNICAMP

Comunicamos que o Protocolo de Pesquisa referente ao Projeto:

Título do Projeto de Pesquisa:
Efeito de íons divalentes na atividade das metaloproteases secretadas por fibroblastos gengivais.

Pesquisador Orientador/Orientado:
Prof. Dr. Sérgio Roberto Peres Line/ Ana Paula de Souza

apresentado a este Comitê para análise ética, segundo a Resolução CNS 196/96, do Conselho Nacional de Saúde, de 10/10/96, foi considerado:

[X] Aprovado.

[ ] Aprovado com pendência, devendo o Pesquisador encaminhar as modificações sugeridas em anexo para complementação da análise do Projeto.

[ ] Com pendência.

[ ] Reprovado.

Análise e parecer do relator:

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