Faculdade de Odontologia de Piracicaba

FOP-UNICAMP

ANA PAULA DE SOUZA PARDO

ANÁLISE DE POLIMORFISMO NO PROMOTOR DO GENE DA METALOPROTEASE DA MATRIZ-1, -2, -9 E DO FATOR TRANSFORMADOR DO CRESCIMENTO-β1: CORRELAÇÃO COM A SEVERIDADE DA DOENÇA PERIODONTAL CRÔNICA

Tese apresentada à Faculdade de Odontologia de Piracicaba Universidade Estadual de Campinas, para a obtenção do título de Doutor em Biologia Buco-Dental, área de Histologia e Embriologia

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A Comissão Julgadora dos trabalhos de Defesa de Tese de DOUTORADO, em sessão pública realizada em 24 de Outubro de 2002, considerou a candidata ANA PAULA DE SOUZA PARDO aprovada.

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Antoine de Saint-Exupéry

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SUMÁRIO

RESUMO

Metaloproteases da matriz (MMPs) degradam muitas das proteínas que compõe a matriz extracelular, estando sua atividade implicada com a destruição do tecido conjuntivo durante a invasão do câncer, destruição da cartilagem na artrite, ruptura da placa aterosclerótica e destruição dos tecidos periodontais na periodontite. Recentemente, variações genéticas foram detectadas na região do promotor do gene de várias MMPs. Esses polimorfismos genéticos têm apresentado alelos-específicos que afetam a atividade de transcrição do promotor do gene das MMPs, podendo estar associados com susceptibilidade e/ou severidade a doenças comuns e complexas. Similarmente, polimorfismos genéticos foram encontrados no promotor do gene do Fator Transformador do Crescimento (TGF-β_1), um importante fator responsável pelo aumento na síntese de colágeno. Estas alterações poderiam contribuir com o descontrole entre síntese e destruição dos componentes da matriz extracelular. Atualmente, a doença periodontal representa o principal fator responsável pela perda de dentes em adultos. O papel das bactérias na iniciação da doença está bem documentado, mas a ativação das células de defesa requeridas para produção e liberação de mediadores que estimulam os mecanismos de destruição do tecido conjuntivo não é claro. Sendo assim, o objetivo deste estudo foi detectar polimorfismos genéticos no promotor do gene da MMP-1, MMP-2, MMP-9 e TGF-β1 e associar à susceptibilidade e/ou severidade da doença periodontal crônica em uma população Brasileira.

ABSTRACT

Matrix metalloproteinases (MMPs) degrade a range of extracellular matrix proteins and have been implicated in connective tissue destruction during cancer invasion, cartilage destruction in arthritis, atherosclerotic plaque rupture, and periodontal tissues destruction. Recently, genetic variances have been detected in the promoter of a number of MMPs genes. These genetic polymorphisms have been shown to have allele-specific effects on the transcription activities of MMP gene promoters, and to be associated with susceptibility and/or severity to several diseases. Similarly, genetic polymorphisms have been found in the gene promoter of the transforming growth factor-β1 (TGF-β1), an important growth factor reponsible to increase the collagen production. These alterations could contribute to the imbalance between the synthesis and degradation of the extracellular matrix components. Presently, periodontal disease represents the main factor responsible for loss of teeth in adults. The role of bacterial in the initiation of periodontitis is very well documented, but the activation of the host defense cells requeired to release mediators that stimulate the pathway connective tissue breakdown is unclear. Likewise, the aim of our study was to detect genetic polymorphisms in the gene promoter of MMP-1, MMP-2, MMP-9, and TGF-β_1 and to associate with susceptibility and/or severity of the chronic periodontal disease in a Brazilian population.

INTRODUÇÃO

A periodontite representa uma doença inflamatória que afeta os tecidos componentes do periodonto, um órgão com características anatômicas e fisiológicas peculiares, que está exposto a um microambiente onde a ameaça bacteriana pode ser muito variável. Esta patologia é relativamente comum em adultos após a terceira década de vida, onde se caracteriza por uma inflamação crônica e de progressão lenta.

A atividade de metaloproteases da matriz nos tecidos periodontais representa a real via efetora da destruição tecidual. A síntese destas enzimas é influenciada por citocinas inflamatórias e fatores do crescimento, sendo o controle da atividade regulado por proteínas teciduais inibidoras de metaloproteases (TIMP) na matriz extracelular.

A compreensão da patogênese da periodontite ainda é muito limitada, estando a terapia voltada para o controle da causa mais aceita, a placa bacteriana. Entretanto, este fator parece desencadear uma série de eventos celulares e moleculares que ocorrem no periodonto, onde a resposta do hospedeiro ao ataque bacteriano deve ser o principal responsável pelo grau de severidade da periodontite.

Alguns fatores modificadores que não causam a doença periodontal podem amplificar os mecanismos que a tornam mais severa. O cigarro, o estresse psicossocial, certas doenças sistêmicas e determinados polimorfismos genéticos são exemplos destes fatores que tornam os indivíduos mais suscetíveis à progressão e à severidade da doença periodontal. Polimorfismos são variações genéticas

encontradas na população, estando dentro de um padrão de normalidade, mas que podem tornar um indivíduo mais ou menos suscetível a uma determinada patologia.

METALOPROTEASES DA MATRIZ, TIMP e TGF-β_1

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 da família das metaloproteases da matriz e dos ativadores de plasminogênio são os principais reguladores da degradação da matriz extracelular (Birkedal-Hansen, 1993).

As metaloproteases da matriz (MMPs) compreendem uma família de enzimas que apresentam especificidade por macromoléculas que compõe a matriz extracelular. A família das metaloproteases é formada por pelo menos vinte membros que exibem similaridades estruturais e funcionais. Estas enzimas são secretadas na forma inativa, como zimógeno e/ou como complexo enzima-inibidor (Emonar e Grimaud, 1990; Stricklin et al., 1983). A ativação se dá em duas etapas. Inicialmente o zimógeno sofre clivagem proteolítica por várias enzimas como a tripsina, plasmina, catepsina B e elastase (Van Wart e Birkedal-Hansen, 1990), que resulta na remoção da porção amino-terminal. Em uma segunda etapa a enzima sofre autodigestão que resulta na sua forma ativa. 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

resíduos de histidina presente no domínio catalítico da molécula têm importância crucial para o funcionamento adequado das metaloproteases (Souza *et al.*, 2000). Os dois átomos de cálcio conferem uma estabilidade para a estrutura terciária da proteína (Dioszegi et al., 1995).

As metaloproteases da matriz desempenham papel importante em vários processos fisiológicos e patológicos, como na involução pós-parto (Weeks et al., 1976), 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-Harmainen et al.,1993) e na invasão e metástase de tumores malígnos (Declerck et al., 1992).

As MMPs estão divididas em 3 importantes classes: 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, distribuição, estrutura química e molecular em processos normais e patológicos. Estas enzimas são as únicas com capacidade de clivar a tríplice hélice dos colágenos tipo I, II, III em condições fisiológicas, tornando estas moléculas suscetíveis 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 MMP-1, que exibe uma distribuição ambígua, é sintetizada por fibroblastos, queratinócitos, células endoteliais, macrófagos, osteoblastos e condrócitos (Bikedal-Hansen, 1993). Quando analisada em

eletroforese de gel de poliacrilamida, a colagenase de fibroblastos aparece como várias bandas com peso molecular variando entre 57 e 52 kDa. Esta variação provavelmente se deve à proteólise parcial da molécula. A MMP-8 é produzida exclusivamente por leucócitos polimorfonucleares. Apesar de possuir um núcleo protéico muito semelhante à colagenase de fibroblastos, a alta taxa de glicosilação faz com que esta molécula tenha peso molecular de 75 kDa. Ao contrário da colagenase de fibroblasto, que é rapidamente secretada após a sua síntese, a colagenase de polimorfonuclear 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 utilizadas em laboratório devido a sua grande especificidade a tripla hélice da molécula do colágeno.

As gelatinases 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 sofreu ação de outras MMPs (Reponen et al., 1992 e Reponen et al., 1994). A gelatinase de 72 kDa (MMP-2) 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 abundantemente 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 (core protéico), colágeno tipo IV, V, VII e X, colágeno desnaturado (gelatina) e caseína (Chin et al., 1985). Quando analisada em gel de poliacrilamida, apresenta um peso 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 peso molecular semelhante à estromelisina 1 e parece não responder a fatores de crescimento (Birkedal-Hansen, 1993).

Os genes que transcrevem as MMPs respondem em níveis de transcrição a diferentes fatores do crescimento e citocinas inflamatórias. O TGF-β_1 (Fator Transformador do Crescimento, membro da super-família TGF-β_s) é aparentemente um dos mais importante fatores do crescimento reguladores da síntese de MMPs em fibroblastos. Este fator atua como estimulante da atividade reparadora nos tecidos, inibindo a expressão, síntese e liberação de MMPs pelos fibroblastos, além de estimular a produção de TIMP (inibidor tecidual de metaloprotease) que neutraliza a atividade da enzima na matriz extracelular. Classicamente o TGF-β_1 é considerado um fator promotor de fibrose. Entretanto, alguns autores classificam o TGF-β_1 como um fator pró-inflamatório, uma vez que ele atua como um agente

quimiotático para neutrófilos, monócitos e linfócitos do sangue (Torre-Amione *et al.*, 1990).

A regulação da atividade proteolítica das metaloproteases ocorre a nível extracelular através de proteínas inibidoras específicas de tecido, os TIMPs (inibidor tecidual de metaloprotease da matriz). Três membros desta família têm sido identificados: TIMP-1, TIMP-2 e TIMP-3 (Herron et al., 1986; Woessner 2002). 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 do TIMP é complexo, envolve numerosos pontos de interação com as metaloproteases. O evento principal parece ser resultado da ligação ao domínio catalítico N-terminal, do sítio ativo da metaloprotease (Murphy et al., 1994). O 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). Alterações nos níveis de síntese entre TIMPs e MMPs pode levar a um desequilíbrio na taxa de degradação da matriz extracelular, podendo causar destruição anormal da matriz (Macnaul et al., 1990).

METALOPROTEASES DA MATRIZ E A DESTRUIÇÃO TECIDUAL NA DOENÇA PERIODONTAL

A gengivite representa o início da lesão, caracterizado pela inflamação do tecido de sustentação que circunda os dentes devido ao acúmulo de placa dental bacteriana. A destruição dos tecidos tem início após três a quatro dias de acúmulo de placa bacteriana na superfície dental, estando associada com a migração de neutrófilos (polimorfonucleados do sangue) para a região do epitélio juncional e do 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 MMPs armazenadas nos grânulos citoplasmáticos dos neutrófilos (Souza *et al.*, 1998). Os neutrófilos e os macrófagos são as mais importantes células responsáveis pela destruição do periodonto. Alguns autores têm demostrado que a doença periodontal ocorre em ciclos recorrentes, alternando entre períodos de destruição do tecidual e períodos de estabilidade. Esta teoria relaciona as perdas com os períodos de ativação de MMPs liberadas pelos neutrófilos na matriz extracelular do periodonto e os períodos de estabilidade com o tempo necessário para a síntese de novas enzimas após a exaustão das proteases contidas nos grânulos citoplasmáticos (Van der Zee e Burtsen, 1997).

Alterações qualitativas e quantitativas ocorrem nos colágenos gengivais durante a periodontite. Na gengiva o colágeno se torna mais solúvel devido a presença de inúmeras fibrilas de colágeno que não chegam a se polimerizar em fibras e são degradadas, indicando síntese de novo colágeno ou de colágeno deficiente (Bartold *et al.*, 1996).

Duas vias estão implicadas com a degradação fisiológica e patológica do colágeno periodontal. A primeira ocorre através da fagocitose de fibrilas do colágeno pelos fibroblastos, após colagenases sintetizadas por este tipo celular terem clivado o colágeno tipo I. A segunda via está relacionada com a degradação do colágeno pela exacerbação na síntese de MMPs, associada com o processo inflamatório. Ambas as vias são mediadas pela ação de fatores do crescimento e citocinas da inflamação (Birkedal-Hansen, 1993), estando a via efetora da destruição sempre relacionada com a atividade de MMPs.

O valor dos parâmetros clínicos tradicionalmente usados na determinação do prognóstico da doença periodontal parece estar pelo menos em parte relacionado com genótipos específicos. São recentes as primeiras descrições de polimorfismos gênicos associados à doença periodontal. Polimorfismos nos genes da IL-1α e β e TNF-α foram os primeiros a serem associados com a periodontite, identificando indivíduos suscetíveis à severidade da doença periodontal crônica (Kornman *et al.*, 1997; Gore *et al.*, 1998). Foi demonstrado estatisticamente, em um estudo longitudinal de 14 anos, que a presença de genótipos variantes (chamados positivos para IL-1), resultantes de polimorfismos, aumenta as chances de morbidade dental em 2,7 vezes e, quando associado ao efeito do fumo, pode resultar em risco de até 7,7 vezes maior de perda dental (McGuire e Nunn, 1999).

A associação entre uma dada doença e uma variação genética conhecida, além de permitir a identificação de indivíduos de maior risco ao desenvolvimento da doença em questão, pode contribuir com o melhor entendimento da patogênese da doença periodontal (Sofaer, 1990).

MMP-1 promoter polymorphism: Association with chronic periodontitis severity in a Brazilian population

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Short tittle: MMP-1 polymorphism and chronic periodontitis

Abstract

Background: A single nucleotide polymorphism was described in the promoter region of the human MMP-1 gene, and this polymorphism has been associated with risk of cancer metastasis and inflammatory diseases. In this paper, we studied the possible relationship between the MMP-1 promoter polymorphism and the severity of chronic periodontitis.

Methods: Genomic DNA from oral mucosa was amplified by PCR and analyzed by restriction endonuclease. The alleles were separated by polyacrylamide gel electrophoresis. The significance of the differences in observed frequencies of polymorphism in moderate and severe disease and healthy groups was assessed by Chi-squared test.

Results: In the healthy group, the 2G allele was observed with a frequency of 48.7%, while in severely diseased patients the 2G allele was seen at 69.2% (p =0.0344). The genotype 2G/2G was found at 46.15% in the group with severe periodontitis, and the frequencies of 24.3% and 25.0%, respectively in healthy and moderate group (p =0.0647).

Conclusion: These results show that a polymorphism in the promoter region of MMP-1 gene is associated with the severe chronic periodontitis phenotype in non-smokers.

Key words: MMP-1; polymorphism; chronic periodontitis severity

Periodontal disease (PD) is characterized by inflammatory cell accumulation in the periodontal tissues. Since the majority of pathogenic bacteria resides in periodontal pockets and does not invade the periodontal tissues, the immune system can never efficiently eliminate the microorganisms (Okada & Murakami 1998). This unique situation leads to a chronic inflammation and continuous host response, resulting in tissue destruction (Page 1992, Genco 1992).

The degradation of periodontal tissues is mainly mediated by matrix metalloproteinases (MMPs), which is a family of metal-dependent proteolytic enzymes that mediate the degradation of extracellular matrix and basement membranes. Metalloproteinases 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 (Murphy & Knauper 1997). 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 (Murphy & Knauper 1997, Barlett et al. 1996). Many types of MMPs have been identified in inflamed periodontal tissues and these enzymes are thought to play an important role in tissue destruction in periodontal diseases (Birkedal-Hansen 1993, Ingman et al. 1996). Fibroblast-type collagenase (MMP-1) is the major type of proteolytic enzyme that can cleave native interstitial collagens type I and III, which are the most abundant protein components of periodontal extracellular matrix.

A polymorphism in the promoter region of human MMP-1 gene has been described (Rutter et al. 1998). The two alleles (1G and 2G) are formed by an insertion/deletion

of a guanine at position –1607. The 2G allele has been shown to significantly increase the transcriptional activity (Rutter et al. 1998). Tumors bearing the 2G allele can secrete higher levels of MMP-1, and the presence of this allele was associated with the development of ovarian cancer (Kanamori et al. 1999).

Current concepts of the etiology of chronic periodontitis implicate bacterial infection by gram-negative organisms as the primary cause of disease (Flemming 1999). However, the simple presence, type or quantity of infection do not explain the different evolution and the poor prognosis in severe chronic periodontitis. Therefore, the influence of host genetic factors in disease progression was suggested by some authors (Michalowicz et al. 1991, Offenbacher 1996). Kornman et al. (1997) reported the association between IL-1 genotype and periodontitis severity, encouraging the search for some specific genetic markers for periodontitis (Michalowicz et al. 2000). The purpose of this study was to investigate the presence of the polymorphism 1G/2G in the promoter of MMP-1 gene in individuals with different levels of chronic periodontal disease in order to verify a possible relationship between this MMP-1 polymorphism and chronic periodontitis.

Material and Methods

Subject selection

A convenience sample of 87 unrelated, non-smoking Caucasian subjects >25 years of age, were recruited for study from the patient pool at the Dental Clinics of the Faculty of Dentistry of Piracicaba – UNICAMP. The patients are from the Southeastern region of Brazil. The baseline clinical parameters for the subject population are presented in Table 1. All subjects were in good general health and had

at least 20 teeth in the mouth. Subjects did not have any of the following exclusion criteria: diseases of the oral hard or soft tissues except caries and periodontal disease; use of orthodontic appliances; need for pre-medication for dental treatment; chronic usage of anti-inflammatory drugs; a history of diabetes, hepatitis or HIV infection; immunosuppressive chemotherapy; history of any disease known to severely compromise immune function; present acute necrotizing ulcerative gingivitis or current pregnancy or lactation. Subjects completed personal medical and dental history questionnaires, and within a protocol approved by an Institutional Review Board, signed a consent form after being advised of the nature of the study. Diagnosis and classification of disease severity were made on the basis of clinical parameters and consisted of physical examination, medical and dental history, probing depth, assessment of attachment loss (CAL), tooth mobility and observation of bleeding on probing. Measurements of probing depth and attachment level were recorded at 6 points around each tooth. Subjects were included in clinical categories according to PD severity:

1) *Healthy group*: Subjects found to exhibit no signs of periodontal disease as determined by the absence of clinical attachment loss and no sites with probing depth >3 mm together with less than 10% of sites with gingivitis upon clinical examination (n = 37)
2) *Moderate Periodontitis*: Patients with teeth exhibiting ≥ 3 mm and <7 mm CAL (n = 24)
3) *Severe Periodontitis* Patients with teeth exhibiting ≥7 mm CAL (n = 26)

Sampling

The sampling of epithelial buccal cells was performed as described by Trevilatto & Line (2000). Briefly, 87 individuals undertook a mouthwash after 1 min, containing 5 ml 3% glucose. Following mouthwash, a sterile wood spatula was used to scrape oral mucosa. The tip of the spatula was then shacked into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000 rpm for 10 min. The supernatant was discarded and the cell pellet resuspended in 500 μl of extraction buffer [10 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5% SDS]. The samples were then frozen at -20°C until used for DNA extraction.

DNA Extraction

After defrosted, samples were incubated overnight (ON) with 100 ng/ml proteinase K (Sigma Chemical Co., St. Louis, MO, USA) at 37°C with agitation. DNA was then purified by sequential phenol/chloroform extraction and salt/ethanol precipitation. DNA was dissolved in 70 μl TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA]. The concentration was estimated by measurements of OD 260.

Polymerase Chain Reaction

The sequences of PCR primers were 5`-TCGTGAGAATGTCTTCCCATT-3` (forward primer) and 5`-TCTTGGATTGATTTGAGATAAGTGAAATC-3` (reverse primer). PCR was carried out in a total volume of 50 μl, containing 500 ng genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$,1 μM of each primer, 200 mM each dATP, dCTP, dGTP and dTTP, and 4 units Taq DNA polymerase (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The solution was incubated for 1 min at

95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C, with a final extension of 72°C for 7 min.

Restriction endonuclease digestion

A 15-μl aliquot of PCR products was mixed with a 5 μl solution containing 2 μl 10x NE Buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9), 0.2 μl bovine serum albumin (10 mg/ml), 0.3 μl *Xmnl* (20 units/ml) (New England Biolabs, Inc., Beverly, MA, USA) and 2.5 μl sterile deionized H_2O. The solution was incubated at 37°C ON.

Gel electrophoresis

The total amount aliquot of the digest was mixed with 3 μl of loading buffer and electrophoresed on a 10% vertical non-denaturing polyacrylamide gel at 20 mA. The gel was silver staining by DNA Silver Staining Kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Statistical Analysis

Differences in genotype distribution from those expected by Hardy-Weinberg equilibrium and the significance of the differences in observed frequencies of the polymorphism in moderate and severe disease and healthy groups was assessed by the Chi-square test. $P \leq 0.05$ was considered statistically significant. The risk associated with individuals alleles and genotype was calculated as the Odds ratio (OR) with 95% confidence intervals.

Results

Two mismatches were introduced in the reverse primer annealed to the proximity of the polymorphism (Dunleavey et al. 2000), creating a recognition sequence (5`-GAANNNNTTC-3`) for the restriction endonuclease *XmnI* when the DNA template contains 1G (but not 2G) at the polymorphism site. Thus, *XmnI* digests the 1G allele creating two fragments of 89 bp and 29 bp.

The polymorphism demonstrated Hardy-Weinberg equilibrium ($p>0.05$). There was a small difference in the presence of the different alleles between the healthy, moderate and severe periodontitis groups ($p=0.0607$). However, we have seen a significant difference in the presence of the different alleles when we compared the healthy group versus severe periodontitis group ($p=0.0344$). In the healthy and moderate periodontitis groups, the 2G allele was observed with a frequency of 48.7% and 52.1% respectively, while in severely diseased patients the 2G allele was seen at 69.2%. The frequencies of the different alleles are show in table 2. We have not seen significant difference between the genotypes frequencies in the three different groups ($p=0.2104$). However, when we compare the groups healthy/moderate versus severe periodontitis we observed a difference in the genotypes frequencies between the groups ($p=0.0647$). The genotype 2G/2G was found at 46.15% in the group with severe periodontitis, and the frequencies of 24.3% and 25.0% were observed respectively in healthy and moderate groups. The frequencies of the different genotypes of the MMP-1 gene are shown in table 3. In a separated analysis we grouped individuals healthy and moderate versus severe and calculated the risk associated with individual alleles. Individuals with the 2G allele seem to be approximately twice more likely to develop the severe periodontitis ($p=0.029$;

OR=2.25, 95% CI=1.13– 4.47). Individuals with the 2G/2G genotype seem to be 6 times more likely to develop severe periodontitis than individuals who are 1G/1G homozygous (p=0.0531, OR=6.0, 95% CI=1.1417–31.5326).

Discussion

Chronic periodontitis is the most common form of periodontal disease. It affects over 30% of adult population, with severe disease occurring in 7-13% of adults (Brow & Löe 1993). It is clearly a multifactorial disease, in which disease susceptibility, progression and treatment outcome are dependent on multiple environmental and genetic factors (Engerbretson et al. 1999, Kornman et al. 1997). A recent study using 117 pairs of adult twins estimated that about half of the variance found in the expression of chronic periodontitis can be attributed to genetic factors (Michalowicz et al. 2000). Kornman et al. (1997) were the first to show that a specific genetic variation of the IL-1 gene cluster was associated with the severity of chronic periodontitis. Since then, the search for specific genetic risk factors (i.e. gene polymorphisms) has become a major subject matter in clinical periodontology. Gene polymorphisms are a mechanism by which individuals may exhibit variations within the range of what is considered biologically normal (Komman et al. 1997).

In this study we report a polymorphism in the promoter region of MMP-1 gene as been associated with the severe chronic periodontitis phenotype in non-smokers. The 2G/2G genotype has been found in approximately 30% of the population while the occurrence of heterozygotes (1G/2G) has been of approximately 39% (Rutter et al. 1998). We have observed an association between the presence of 2G/2G genotype of the MMP-1 gene and the severity of periodontal disease in our analysis with

Caucasian subjects. This finding is in accordance with other studies that have correlated the 2G/2G genotype with high risk to develop ovarian cancer (Kanamori et al. 1999) and colorectal cancer invasiveness (Ghilardi et al. 2001). The presence of 2G creates the sequence 5' AAGGAT 3', which is the consensus sequence for the Ets family of transcription factors. This polymorphism was shown to augment transcriptional activity and can potentially increase the levels of protein expression (Rutter et al. 1998). This mechanism provides the molecular bases for a more intense degradation of periodontal extracellular matrix, leading to increased susceptibility to the development of severe periodontitis in adults.

Matrix metalloproteinase-1 seems to play an important role during the destruction of the extracellular matrix in periodontal disease. Immunoreactivity for MMP-1 was found in granulation tissue of chronic periodontitis patients, while only moderate immunostaining for MMP-8 (neutrophil collagenase) could be detected (Ingman et al. 1994). Additionally, reverse transcriptase-polymerase chain reaction has shown that mRNA levels of MMP-1 are significantly increased in inflamed gingival tissue, while only very low levels of MMP-8 transcripts were detected in diseased gingival tissue. (Aiba et al. 1996). These results suggest that MMP-1 rather than MMP-8 seems to be the major interstitial collagenase present in inflamed periodontal tissue.

In conclusion, our results indicate that the 1G/2G polymorphism in the promoter of the MMP-1 gene could be a risk factor for severe chronic periodontitis. This polymorphism can be used as a genetic marker for severe periodontitis, and it is likely that other markers will be reported in the next few years. We believe that the identification of several genetic markers for susceptibility to severe chronic periodontitis will allow a precise and early identification of individuals at high risk.

Besides prevention, the genetic analysis of affected patients will help in the design of individualized forms of therapy and in the prediction of treatment outcome.

Acknowledgments

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Table 1.

Baseline clinical parameters of the subject population (n=87)

	Healthy (n=37)	Moderate (n=24)	Severe (n=26)
AGE (years) Mean (± SD)	43.7 (±14.5)	37.9 (±11.2)	44.2 (±11.2)
GENDER %			
Female	70.3	75.0	84.6
Male	29.7	25.0	15.4

Table 2.

Distribution of the MMP-1 alleles in healthy, moderate and severe groups with chronic periodontitis

Allele	Healthy		Moderate		Severe		*p* Value	OR
	n	%	n	%	n	%		(95%)
1G	38	51.3	23	47.9	16	30.8	0.0607	2.25
2G	36	48.7	25	52.1	36	69.2		

Table 3.

Distribution of the MMP-1 genotype in the healthy group, and in groups with moderate and severe chronic periodontitis

Genotype	Healthy		Moderate		Severe		*p* Value
	n	%	n	%	n	%	
1G/1G	10	27.0	05	20.8	02	7.70	0.0647
2G/2G	09	24.3	06	25.0	12	46.15	
1G/2G	18	48.7	13	54.2	12	46.15	

ANALYSIS OF THE TGF-β_1 PROMOTER POLYMORPHISM (C–509T) IN PATIENTS WITH CHRONIC PERIODONTITIS.

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Short tittle: TGF-β_1 promoter polymorphism and chronic periodontitis

Abstract

Background: A polymorphism in the promoter region of the TGF-β_1 gene was described at position –509. This polymorphism represents a C-to-T base exchange, which creates a YY1 consensus sequence in an area involved with down transcription regulation. This polymorphism has been associated with risk for asthma and allergies. In this study we have investigated the association between this polymorphism and chronic periodontitis severity.

Methods: Genomic DNA from oral mucosa of 87 Caucasian subjects was amplified by PCR, and digested with *Eco81I* restriction endonuclease. The alleles were separated by polyacrylamide gel electrophoresis. The differences in genotype distribution from those expected by Hardy-Weinberg equilibrium, and the significance of the differences in observed frequencies of the polymorphism in moderate and severe disease and healthy groups was assessed by Chi-square test.

Results: There was difference in the presence of the different alleles and genotypes among the healthy, moderate and severe periodontitis groups. The allele T was seen at 57.7% in the group with severe periodontitis and 37.8% and 35.4% in the healthy group and moderate periodontitis, respectively (p=0.0387). The genotype T/T was found at 38.5% in the group with severe periodontitis, and at a frequency of 8% in the healthy group (p =0.0258).

Conclusion: These results demonstrate that the polymorphism at bp –509 in the TGF-β_1 promoter may have a small effect on the modulation of the inflammatory process during periodontitis.

Key words: TGF-β_1; polymorphism; chronic periodontitis severity; restriction enzyme

Presently, periodontal disease is the main factor responsible for loss of teeth in adults (Wahl et al. 1993), and the role of bacteria in the initiation of periodontitis is well documented (Page 1991, Flemmig 1999). However, the activation of host defense cells, which are required to release mediators that stimulate the pathway connective tissue breakdown is unclear (Page 1991, Genco 1992).

The major cell types in inflamed and healthy periodontal tissues are capable to produce a variety of important pro-inflammatory and anti-inflammatory cytokines and growth factors (Birkedal-Hansen 1993), which mediate the host response. Transforming growth factor beta-1 (TGF-β_1) regulates cell growth, differentiation and function. It is a multifunctional cytokine known to induce the expression of collagen genes and to provoke the extracellular matrix fibrosis (Kim et al. 1989). Particularly, TGF-β_1 is known to have a potent immunosuppressive activity, down-regulating the transcription of other pro-inflammatory cytokines, including interleukin (IL-1), tumor necrosis factor-α (TNF-α) (Musso et al. 1990), and several metalloproteinases (MMPs) (Steinsvoll 1999, Page 1991, Birkedal-Hansen 1993).

Genetic polymorphisms in the TGF-β_1 gene promoter were shown to interfere with the transcriptional activity of this gene (Hobbs et al. 1998, Kim et al. 1989). The C-to-T polymorphism at –509 position of the human TGF-β_1 promoter creates two different alleles located in a region that is thought to be a negative regulatory area of TGF-β_1 gene (Hobbs et al. 1998). This polymorphism creates a YY1 activator consensus sequence (Shrivastava & Calame 1994). It is associated with risk for asthma and allergies, promoting the increase of the levels of serum IgE, and susceptibility to osteoporosis (Yamada et al. 2001). However, the precise effect of T or C alleles on the transcriptional activity of TGF-β_1 gene is not known.

Kornman et al. (1997) were the first to report the association between a IL-1 genotype composite and periodontal disease. A number of other studies have also focused attention to the association of genetic polymorphisms and the susceptibility to periodontal disease (Michalowicz et al. 2000, Socransky et al. 2000, Engebretson et al. 1999, Diehl et al.1999), encouraging the search for genetic markers for periodontitis. The purpose of our study was to investigate the frequencies of the –509 polymorphism alleles and genotypes in the TGF-β_1 gene promoter in individuals with different levels of chronic periodontal disease.

Material and Methods

Subject Selection

A convenience sample of 87 unrelated, non-smoking Caucasian subjects >25 years of age, were recruited for study from the patient pool at the Dental Clinics of the Faculty of Dentistry of Piracicaba – UNICAMP. The patients are from the Southeastern region of Brazil. The baseline clinical parameters for the subject population are presented in Table 1. All subjects were in good general health and had at least 20 teeth in the mouth. Subjects did not have any of the following exclusion criteria: diseases of the oral hard or soft tissues except caries and periodontal disease; use of orthodontic appliances; need for pre-medication for dental treatment; chronic usage of anti-inflammatory drugs; a history of diabetes, hepatitis or HIV infection; immunosuppressive chemotherapy; history of any disease known to severely compromise immune function; present acute necrotizing ulcerative gingivitis or current pregnancy or lactation. Subjects completed personal medical and dental history questionnaires, and within a protocol approved by an

Institutional Review Board, signed a consent form after being advised of the nature of the study. Diagnosis and classification of disease severity were made on the basis of clinical parameters and consisted of physical examination, medical and dental history, probing depth, assessment of attachment loss (CAL), tooth mobility and observation of bleeding on probing. Measurements of probing depth and attachment level were recorded at 6 points around each tooth. Patients were carefully examinated in order to avoid discrepancy among the periodontal status of the patients. Subjects were included in clinical categories according to PD severity:

1) *Healthy group*: Subjects found to exhibit no signs of periodontal disease as determined by the absence of clinical attachment loss and no sites with probing depth >3 mm (n = 37)
2) *Moderate Periodontitis*: Patients with at least three teeth with sites exhibiting ≥ 3 mm and <7 mm CAL, at least two quadrants (n = 24)
3) *Severe Periodontitis* Patients with at least three teeth exhibiting sites ≥7 mm CAL, at least two quadrants (n = 26). All patients of this group had other teeth exhibiting signs of periodontal disease with probing depth <7 mm.

Sampling

The sampling of epithelial buccal cells was performed as described by Trevilatto & Line (2000). Briefly, 87 individuals undertook a mouthwash after 1 min, containing 5 ml 3% glucose. Following mouthwash, a sterile wood spatula was used to scrape oral mucosa. The tip of the spatula was then shacked into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000 rpm for 10 min. The supernatant was discarded and the cell pellet resuspended in 500 μl of

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DNA Extraction

After defrosted, samples were incubated overnight (ON) with 100 ng/ml proteinase K (Sigma Chemical Co., St. Louis, MO, USA) at 37°C with agitation. DNA was then purified by sequential phenol/chloroform extraction and salt/ethanol precipitation. DNA was dissolved in 70 μl TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA]. The concentration was estimated by measurements of OD 260.

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The sequences of PCR primers were 5`-TTTTGCCATGTGCCCAGTAG -3` (forward) and 5`-CACCAGAGAAAGAGGACCAG -3` (reverse). PCR was carried out in a total volume of 50 μl, containing 500 ng genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1 μM of each primer, 200 mM each dATP, dCTP, dGTP and dTTP, and 2.5 units *Taq* DNA polymerase (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The solution was incubated for 3 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 57°C and 1 min at 72°C, with a final extension of 72°C for 7 min.

Restriction endonuclease digestion

A 15-μl aliquot of PCR products was mixed with a 5 μl solution containing 2 μl 10x NE Buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9), 0.1

μl *Eco 81I* (20 units/ml) (New England Biolabs, Inc., Beverly, MA, USA) and 2.5 μl sterile deionized H_2O. The solution was incubated at 37°C ON.

Gel electrophoresis

The total amount aliquot of the digest was mixed with 3 μl of loading buffer and electrophoresed on a 10% vertical non-denaturing polyacrylamide gel at 20 mA. The gel was silver stained by DNA Silver Staining Kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Statistical analysis

Differences in genotype distribution from those expected by Hardy-Weinberg equilibrium and the significance of the differences in observed frequencies of the polymorphism in moderate and severe disease and healthy groups was assessed by the Chi-square test (χ^2), and in certain analysis by the Fisher exact test. $P< 0.05$ was considered statistically significant.

Results

Table 1 shows the baseline clinical parameters of the subject population. We have found a difference in the presence of the different alleles between the healthy, moderate and severe groups (p =0.0387, χ^2) (Table 3). The strongest difference was observed between healthy *versus* severe groups (p =0.0398, Fisher exact test). The same was observed in the presence of the different genotypes between the groups (p=0.0258 by χ^2) (Table 2). The T allele was found at a frequency of 57.7% in the severe group, while in the healthy and moderate groups the T allele was seen at

37.8% and 35.4% respectively. The genotype T/T was found at 38.5% in the group with severe periodontitis, in the healthy and moderate groups the frequency was 8.0% and 12.6% respectively.

Discussion

Gene polymorphisms are a mechanism by which individuals may exhibit variations within the range of what is considered biologically normal. Actually, the study of new genetic polymorphisms has become a field for new insights in periodontology, once there are several inflammatory cytokines and growth factors involved with the complex mechanisms of periodontal disease. We are interested in the role of these genetic variances in chronic periodontal disease and have studied the C-to-T polymorphism at –509 position of the human TGF-β_1 promoter. The T/T genotype was found in a higher frequency in the severe than in the control and moderate groups. Additionally, the T allele was also found in a higher frequency in the severe group. The frequency distribution of TGF-β_1 alleles, while demonstrating an approximation of Hardy-Weinberg distribution, was not in equilibrium for TGF-β_1. These findings could indicate that TGF-β_1 may have a small influence on the severity of the chronic periodontal disease. This fact is not unexpected since periodontitis is a multifactorial disease whose development is dependent on several genetic and environmental factors. Additionally, transforming growth factor-β_1 has been described as a cytokine with pleiotropic properties (Steinsvoll et al. 1999). In the periodontium, TGF-β_1 suppresses proteolytic activity by down-regulating the transcriptional activity of matrix metalloproteinases (MMPs) and activates the expression of tissue inhibitors of MMPs (Overall et al. 1991, Birkedal-Hansen 1993).

It can also stimulate the synthesis of connective tissue matrix components by fibroblasts and osteoblasts (Overall et al. 1991). This results in the formation of extracelular matrix and induces fibrosis (Page 1991, Hobbs et al. 1998). On the other hand, TGF-β_1 was shown to induce the chemotaxis for neutrophils, monocytes (Wahl et al. 1993), mast cells and lymphocytes. It may also augment the release of leukocyte cytokines, which could contribute to the breakdown of periodontal tissue, especially in the early phases of the inflammatory response (Wahl 1992, Wahl et al. 1993). This bifunctional role of TGF-β_1 may explain a small association found when comparing the genotypes of control and severe groups.

The control mechanisms of the TGF-β_1 concentration in plasma are poorly understood. Evidences show that the concentration of active TGF-β_1 may be predominantly under genetic control. Grainger et al. (1999) have observed that the C-509T polymorphism is significantly associated with the TGF-β_1 plasma concentration. A significant association between the C-509T polymorphism and bone mineral density was detected in a study containing 625 postmenopausal Japanese women. The genotype T/T was found in higher frequency in 286 individuals with osteoporosis than in 170 normal controls. The frequencies of the CC, CT, and TT genotypes of the C-509T polymorphism in the studied population were 24%, 49%, and 27%, respectively (Yamada et al. 2001). However, other authors have seen that this polymorphism is not associated with coronary artery disease, and its presence alone would not be a genetic risk factor for predisposition to heart diseases (Syrris et al. 1998).

Relating genetic polymorphisms to diseases is a hard task, once it is given that there are around ten distinct single nucleotide polymorphisms for every 10 kb in the

human genome, of which most of them are considered silent (Hennig et al. 1999) Studies have reported that some cytokines, such as TNF-α, IL-1α, IL-1β, and IL-6 are encoded by polymorphic genes, showing genotypes associated with inflammatory diseases, that may confer susceptibility to periodontal disease (Kornman et al. 1997, Gore et al. 1998, Diehl et al. 1999, Morse et al. 1999, Engerbretson et al. 1999). These molecules contribute to the destruction of type I collagen, and also promote bone resorption by stimulating proliferation, differentiation and activation of osteoclasts (Wilton et al. 1992). Kornman et al. (1997) have proposed that the IL-1-α and -β composite genotype would be associated with the severity of periodontal disease. The composite genotype (IL-α^{-889} allele 2 in conjunction with IL-$1\beta^{+3953}$ allele 2) occurred in 78% of the individuals with severe periodontitis (Kornman et al. 1997). Genotype 2/2 of IL-1β +3953 polymorphism has been associated with a 4-fold increase in IL-1β production (Gore et al. 1998, Trevilatto et al. 2001). Likewise, other genetic variations have been found in the promoter region of TGF-β_1 and studies are required in order to verify the association of these TGF-β_1 polymorphisms with the periodontal disease progression.

In summary, we have seen that the polymorphism at bp –509 in the TGF-β_1 promoter may have a small effect on the modulation of the inflammatory process of periodontitis. However, the increasing interest to find genetic markers for periodontal disease is essential. Likewise, it is important to determine the exact function of these genetic polymorphisms during periodontitis in order to know their exact weight during

the disease progression. It could facilitate the early identification of individuals at high risk, providing the design of new individualized forms of periodontal therapy.

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Table 1.

Baseline clinical parameters of the subject population (n=87)

	Healthy (n=37)	Moderate (n=24)	Severe (n=26)
AGE (years) Mean (± SD)	43.7 (±14.5)	37.9 (±11.2)	44.2 (±11.2)
GENDER %			
Female	70.3	75.0	84.6
Male	29.7	25.0	15.4

Table 2.

Distribution of the TGF-β_1 genotypes in the healthy group, and in groups with moderate and severe chronic periodontitis

Genotype	Healthy		Moderate		Severe		*p* Value
	n	(%)	n	(%)	n	(%)	
C/C	12	(32.5)	10	(41.6)	06	(23.0)	0.0258 (χ^2)
T/T	03	(08.0)	03	(12.6)	10	(38.5)	
C/T	22	(59.5)	11	(45.8)	10	(38.5)	

Table 3.

Distribution of the TGF-β_1 alleles in healthy, moderate and severe groups with chronic periodontitis

Allele	Healthy		Moderate		Severe		*p* Value
	n	(%)	n	(%)	n	(%)	
C	46	(62.2)	31	(64.6)	22	(42.3)	0.0387 (χ^2)
T	28	(37.8)	17	(35.4)	30	(57.7)	

Absence of *MMP-2* gene promoter polymorphism (–1306) in Brazilian Caucasians

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Running title: Absence of MMP-2 polymorphism

ABSTRACT

Matrix metalloproteinase-2 (MMP-2) is an important enzyme involved in physiologic and pathologic events where extracellular matrix components are degraded. As a preferred substrate, MMP-2 degrades type IV collagen that it is found in basement membrane, so that MMP-2 has been implicated with cancer metastasis. The activity of enzyme is controlled at several levels, and the gene transcription represents one of the most important ways. Recently, a single nucleotide polymorphism at position –1306 of the *MMP-2* gene promoter has been described. A C to T change was shown to interfere with the transcriptional activity of the gene. The T allele disrupts a Sp-1-site, promoting a down regulation of the *MMP-2* gene promoter. This allele was found at frequency of 0.26. We have sequenced the *MMP-2* gene promoter in 45 Brazilian Caucasians and in our study sample we have not found the C allele.

KEY WORDS

Matrix metalloproteinase-2; gelatinase A; gene polymorphism

DATABASE:

MMP-2 – OMIM: 120360; GenBank: U96098, AJ298926

INTRODUCTION

Matrix metalloproteinases (MMPs) represent an important family of metal-dependent endopeptidases that are responsible for the degradation of extracellular matrix (ECM) components. At least twenty MMPs have been characterized and all members of this family have zinc and 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. The activity of MMPs is highly controlled in the ECM by specific tissue inhibitors of MMPs (TIMPs), that form a enzyme-inhibitor complex, regulating the enzyme activity.

MMP-2, also known as "gelatinase A", is widely distributed in the organism. It is constitutively expressed by fibroblasts, endothelial cells and osteoblasts. This enzyme is active in the degradation of denatured fibrillar collagens, elastin and several other components of the extracellular matrix (Creemers et al., 1998; Birkedal-Hansen, 1993; Kerkvliet et al., 1999). Matrix metalloproteinase-2 was shown to participate in the breakdown of ECM in disease processes as diverse as tooth caries (Kawasaki and Featherstone, 1997), periodontal disease (Makela et al., 1994), rheumatoid arthritis and cancer metastasis. Additionally, these enzymes can potentate the degradation of extracellular matrix by activating collagenase-3 (MMP-13) and neutrophil collagenase (MMP-8) (Murphy and Knauper, 1997).

A number of studies have focused attention to the association of genetic polymorphisms and the susceptibility to periodontal disease (Michalowicz et al., 2000; Socransky et al., 2000; Engerbretson et al., 1999; Diehl et al., 1999) encouraging the

search for genetic markers for periodontitis. In order to find a possible association between the *MMP-2* gene promoter polymorphism and periodontal disease we aimed to investigate the frequency of C and T alleles in Brazilian Caucasians with different levels of chronic periodontal disease. Surprisingly, our analysis showed that the C allele was not present in our population study.

MUTATION AND POLYMORPHISM

The cytogenetic location of *MMP-2* was mapped to 16q13, and recently the complete sequence of the gene has been reported (Price et al., 2001). This study identified 15 variant loci in the *MMP-2* gene from a panel of 32 individuas. An important SNP functional polymorphism was seen in the promoter of the gene. A C-to-T base change at –1306 position promotes the disruption of the Sp-1-type promoter site (CCACC box), displayed the decrease of the *MMP-2* promoter gene activity with the T allele. Luciferase activity assay demonstrated that cells transfected with –1306C expressed at least two-fold higher luciferase activity than cells transfected with –1306T constructs. This allele was found at frequency of 0.26. Surprisingly, we have sequenced the *MMP-2* gene promoter in 45 Brazilian Caucasians and in our study sample we have not found the C allele. Data referent to Brazilian census indicates that Caucasians represent about 70% of the Southeastern population, having European ascendance in their majority (IBGE, 1995). Our study group was mainly formed by Portuguese, Spanish and Italian descendants, which may differ from the population sample of the original study, which is from the United Kingdom (Price et al., 2001).

BIOLOGICAL SIGNIFICANCE

Matrix Metalloproteinases activity is under strict control at several levels, including the gene transcription as one of the most important steps. Proofing, evidences have demonstrated that a high activity of *MMP* gene promoters may induce abnormal degradation of extracellular matrix components due excess of enzymes in the tissue. Functional SNP polymorphisms have been found in the promoter of different MMPs, including MMP-1, MMP-3, MMP-9 and MMP-12, and they have been associated with disease susceptibility. Studies have demonstrated that an insertion of one guanine base at position –1607 of the MMP-1 promoter increases the transcription of MMP-1, promoting high risk for ovarian cancer. Coronary atherosclerosis represents a disorder whose etiology appears to be highly associated with *MMP-3*$^{(-1612)}$, *MMP-9*$^{(-1562)}$, and *MMP-12*$^{(-82)}$ polymorphism (Ye, 2000).

Many cell types express MMP-2 in normal and pathological conditions. The enzyme takes part during the remodeling of extracellular matrix, having as preferred substrate type IV collagen that is found at basement membrane, so that it has been associated with the dissemination of malignant tumor through tissues. It is also able to degrade denatured type I collagen, playing an important role in tissues where this collagen has an intense turnover, such as periodontium. Works have shown that secreted MMP-2 is concentrated at sites immediately inflammatory involved in the limited proteolysis of critical ECM proteins and subsequent transition to an inflammatory phenotype. Thus, higher expression of *MMP-2* gene contributes to inflammatory disorders. MMP-2 activity is also involved in initiating or regulating cells responses such as proliferation, adhesion and migration events (Yu et al., 1998). It is

believed that MMP-2 is more than a simple extracellular matrix degrader, this enzyme modifies the ECM in a way to favor host cells responses for certain stimuli (Yu et al., 1998).

Thus, finding different alleles that modify the expression of MMP-2 could provide important insights on the pathogenesis of diseases where MMP-2 is involved, indicating individuals with high risk for diseases. It could also bring future perspectives to study association between *MMP-2* gene polymorphism and diseases. Furthermore, the diagnosis of alleles that impute disease risk could be an auxiliary to indicate the use of alternative therapy, as inhibitors of MMPs, which are a promisse of treatment for disease where the MMPs activity is upregulated (Nagase and Brew, 2002; Rudek et al., 2002).

Our results demonstrated the absence of –1306 *MMP-2* gene polymorphism in a Brazilian Caucasians population. We found only the T allele, which is associated with the lower *MMP-2* gene promoter activity. Further studies are necessary in order to confirm the existence of this polymorphism in the *MMP-2* gene promoter and its frequency in different populations.

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Polymorphism (C–1562T) in the *MMP-9* Gene Promoter and Chronic Periodontal Disease

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Running tittle: *MMP9* gene polymorphism and periodontitis

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Polymorphism (C–1562T) in the *MMP-9* Gene Promoter and Chronic Periodontal Disease

Eur J Oral Sci

Abstract Matrix metalloproteinase-9 (MMP-9, also known as gelatinase B) represents a protease released by inflammatory cells that is highly expressed in the tissues during the process of periodontal destruction. The search for the association between polymorphisms in candidate genes for inflammatory disease may be of key importance to understand the mechanisms that involve the loss of periodontal attachment. A C-to-T base change at –1562 creates two different alleles, where the C/T and T/T genotypes promote high activity of the MMP-9 gene promoter, increasing the risk for coronary atherosclerosis and aneurysm. The purpose of our study was to investigate the relationship between the presence of this MMP-9 gene polymorphism and Caucasian individuals with different levels of chronic periodontal disease. The results showed a negative association between them.

Key words: MMP-9 gene polymorphism; periodontal disease

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Introduction

Periodontal disease is characterized by multiple factors, involving the infection by predominately gram-negative anaerobes microorganisms (1) and the host inflammatory response, whose mechanisms have not been totally clear yet. As part of this context, the role of matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases that is able to degrade the most components of extracellular matrix (ECM), have been investigated by several works. They have established very well the importance of these enzymes in the degradation of periodontal tissues during inflammatory process (2, 3). MMP-9 (also known as gelatinase B) is one member of MMP family that is highly expressed during periodontitis (3, 4). This enzyme is expressed by PMN-leukocyte, macrophage, keratinocyte and endothelial cells (2), being active against connective tissue proteins, such as type IV, V, and XI collagens, proteoglycans and elastin (5). It is released by the cells as zymogens and are thought to be activated in the tissue by a cascade of enzymes comprising serine proteases and other MMPs. The MMP-9 activity is under strict control at several levels. Primarily, the expression of MMP-9 is controlled at the transcription level in response to regulatory molecules as tumor necrosis factor-α, interleukin-1, platelet-derived growth factor, and epidermal growth factor (6, 7, 8). The balance between MMP-9 and the specific tissue inhibitor of MMP (TIMP) regulates the activity of enzyme in the ECM.

A number of studies have focused attention to the association of genetic polymorphisms and the susceptibility and severity to periodontal disease (9, 10, 11, 12, 13) encouraging the search for genetic markers for periodontitis. The *MMP-9* gene shows a functional C-to-T single nucleotide polymorphism (SNP) at position –

gene shows a functional C-to-T single nucleotide polymorphism (SNP) at position –1562, which affects transcription and leads to promoter low-activity (C/C) and high-activity (C/T, T/T) genotypes (13). This *MMP-9* gene polymorphism has been associated with high risk for vascular diseases, such as coronary atherosclerosis (14, 15, 16). In the present paper, we aimed to investigate the association between the presence of this *MMP-9* gene polymorphism in relations to Caucasian individuals that shown several levels of chronic periodontal disease.

Material and Methods

Subject Selection

A convenience sample of 85 unrelated, non-smoking Caucasian subjects >25 years of age, were recruited for study from the patient pool at the Dental Clinics of the Faculty of Dentistry of Piracicaba – UNICAMP. The patients are from the Southeastern region of Brazil. The baseline clinical parameters for the subject population are presented in Table 1. All subjects were in good general health and had at least 20 teeth in the mouth. Subjects did not have any of the following exclusion criteria: diseases of the oral hard or soft tissues except caries and periodontal disease; use of orthodontic appliances; need for pre-medication for dental treatment; chronic usage of anti-inflammatory drugs; a history of diabetes, hepatitis or HIV infection; immunosuppressive chemotherapy; history of any disease known to severely compromise immune function; present acute necrotizing ulcerative gingivitis or current pregnancy or lactation. Subjects completed personal medical and dental history questionnaires, and within a protocol approved by an Institutional Review Board, signed a consent form after being advised of the nature of the study.

Diagnosis and classification of disease severity were made on the basis of clinical parameters and consisted of physical examination, medical and dental history, probing depth, assessment of attachment loss (CAL), tooth mobility and observation of bleeding on probing. Measurements of probing depth and attachment level were recorded at 6 points around each tooth. Subjects were included in clinical categories according to PD severity:

1) *Healthy group*: Subjects found to exhibit no signs of periodontal disease as determined by the absence of clinical attachment loss and no sites with probing depth >3 mm (n = 36)
2) *Moderate Periodontitis*: We found patients with at least three teeth exhibiting sites ≥ 3 mm and <7 mm CAL , at least two different quadrants (n = 24)
3) *Severe Periodontitis*: We found patients with at least three teeth exhibiting sites ≥7 mm CAL, at least two different quadrants (n = 25)

Sampling

The sampling of epithelial buccal cells was performed as described by Trevilatto & Line (17). Briefly, 85 individuals undertook a mouthwash after 1 min, containing 5 ml 3% glucose. Following mouthwash, a sterile wood spatula was used to scrape oral mucosa. The tip of the spatula was then shacked into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000 rpm for 10 min. The supernatant was discarded and the cell pellet resuspended in 500 μl of extraction buffer [10 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5% SDS]. The samples were then frozen at -20°C until used for DNA extraction.

DNA Extraction

After defrosted, samples were incubated overnight (ON) with 100 ng/ml proteinase K (Sigma Chemical Co., St. Louis, MO, USA) at 37°C with agitation. DNA was then purified by sequential phenol/chloroform extraction and salt/ethanol precipitation. DNA was dissolved in 70 μl TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA]. The concentration was estimated by measurements of OD 260.

Polymerase Chain Reaction

The sequence from –1809 to –1374 in the *MMP-9* gene promoter was PCR amplified with primers 5`-GCCTGGCACATAGTAGGCCC-3` (forward) and 5`-CTTCCTAGCCAGCCGGCATC -3` (reverse). PCR was carried out in a total volume of 50 μl, containing 500 ng genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1 μM of each primer, 200 mM each dATP, dCTP, dGTP and dTTP, and 2.5 units *Taq* DNA polymerase (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The solution was incubated for 3 min at 95°C, followed by 35 cycles of 1 min at 95°C, 45 s at 65°C and 45 s at 72°C, with a final extension of 72°C for 7 min.

Restriction endonuclease digestion

A 2-μl aliquot of PCR products was mixed with a 8 μl solution containing 1 μl 10x NE Buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9), 0.3 μl *PaeI* (20 units/ml) (New England Biolabs, Inc., Beverly, MA, USA) and 6.7 μl sterile deionized H_2O. The solution was incubated at 37°C ON.

Gel electrophoresis

The total amount aliquot of the digest was mixed with 3 μl of loading buffer and electrophoresed on a 10% vertical non-denaturing polyacrylamide gel at 20 mA. The gel was silver stained by DNA Silver Staining Kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Statistical analysis

Differences in the significance of the differences in observed frequencies of the polymorphism in moderate and severe disease and healthy groups was assessed by the Chi-square test. $P< 0.05$ was considered statistically significant.

Results

Table 1 shows the baseline clinical parameters of the subject population. The results indicated that there is not an associaiton between the presence of the different C and T allele in relation to the different levels of chronic periodontitis (p = 0.6002). The C allele was seen at frequency of 80.5%, 87.5% and 84%, and the T allele was found at frequency of 19.5%, 12.5% and 16% in the healthy, moderate periodontitis and severe periodontitis groups, respectivily (Table 2). The same was found to the different genotypes (p = 0.7409). The T/T genotype, which conferees the increase of the MMP-9 transcription was not observed in the moderate periodontitis and severe periodontitis groups, and only one T/T was seen in the healthy group. The C/T genotype was seen at frequencies of 33.4%, 25%, and 32% in the healthy, moderate periodontitis and severe periodontitis groups, respectivily (Table 3).

Discussion

Gene polymorphisms are a mechanism by which individuals may exhibit variations within the range of what is considered biologically normal. Generally, they are single nucleotide polymorphism (SNP) that occur in a high frequency in the human genome and can affect the transcription level of genes. Various SNP have been found in the gene promoter of several MMPs, and they have been associated with different diseases. Despite studies had demonstrated an association between the presence of this –1562 SNP in the *MMP-9* promoter and diseases that affect heart tissues and vessels, we did not find a relationship between it and the different levels of the chronic periodontal disease. Other study has not also found correlation between this polymorphism and multiple sclerosis (18). The C-to-T exchange at position –1562 results in the loss of binding of a nuclear protein to this region of the *MMP-9* gene promoter, and an increase in trasncriptional activity in macrophage (19).

Matrix metalloproteinase-9 is the most complex MMP family member in terms of protein structure and regulation of its activity (7). In contrast to the other gelatinase (MMP-2 or gelatinase A) that is produced by a wide range of cells, few cell types produce MMP-9 and its production is not constitutive (5, 7). The expression of MMP-9 is controlled primarily at the transcription level, where the promoter of the gene responds to the stimuli of various cytokines and growth factors, as interleukin-1, platelet-derived growth factor, tumor necrosis factor-α, and epidermal growth factor (6, 20). A cascade of activators and tissue inhibitors of MMPs (TIMPs) represent other control pathways, which regulate the activity of this enzyme in the extracellular matrix (2).

Although MMP-9 has a high affinity to type IV collagen, it is through that the enzyme play an important role during the degradation of denatured type I collagen during periodontitis. Neutrophils are the first-line defense leukocytes in the periodontal disease (7). These cells storage large amounts of MMP-9 in their intracellular granules and the rate of degranulation has been appointed as the major factor that determines quiescence and acute phases in the periodontal disease (21). The ration of MMP-9 has been used as a marker of the periodontitis stage (4). Moreover, potent MMP-9 inductors are found in the gingival crevicular fluid of periodontitis patients, such as lipopolysaccharide (LPS) (5, 7), a microbial product derived of the cellular-membrane rupture, and interleukin-8 (IL-8) that is considered the major human neutrophil chemoattractant. Besides, IL-8 induces the release of MMP-9 from cells, and the enzyme cleaves the IL-8 creating a ten-fold more active fragment of the chemokine (7). Therefore, it is very well established that MMP-9 has an important participation in the periodontitis. However, several factors are involved in the expression, activation and inhibition of the enzyme. The genetic control represents only one of these steps. The –1562 polymorphism in the *MMP-9* gene promoter was not associated with susceptibility or severity of the chronic periodontal disease.

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Table 1.

Baseline clinical parameters of the subject population (n=85)

	Healthy (n=36)	Moderate (n=24)	Severe (n=25)
AGE (years) Mean (± SD)	43.7 (±14.5)	37.9 (±11.2)	44.2 (±11.2)
GENDER %			
Female	70.3	75.0	84.6
Male	29.7	25.0	15.4

Table 2.

Distribution of the MMP-9 alleles in healthy, moderate and severe groups with chronic periodontitis

Allele	Healthy		Moderate		Severe		*p* Value
	n	(%)	n	(%)	n	(%)	
C	58	(80.5)	42	(87.5)	42	(84)	0.6002
T	14	(19.5)	06	(12.5)	08	(16)	

Table 3.

Distribution of the MMP-9 genotypes in the healthy group, and in groups with moderate and severe chronic periodontitis

Genotype	Healthy		Moderate		Severe		*p* Value
	n	(%)	n	(%)	n	(%)	
C/C	23	(63.8)	18	(75)	17	(68)	0.7409
C/T	12	(33.4)	06	(25)	08	(32)	
T/T	01	(02.8)	0.0	(0.0)	0.0	(0.0)	

CONCLUSÕES

A inserção/deleção de uma base guanina na posição –1602 do promotor do gene da metaloprotease-1 (MMP-1) cria o alelo 2G que foi encontrado com maior freqüência nos pacientes que apresentam doença periodontal severa. Assim, nós concluímos que a presença deste alelo em heterozigose ou homozigose (genótipos 1G/2G e 2G/2G) se relaciona com a severidade da doença periodontal crônica.

Foi observado que a presença da base T na posição –509 do promotor do gene do Fator Transformador do Crescimento-β1 (TGF-β1) ocorre com freqüência maior em pacientes que apresentam periodontite crônica severa, relacionando os genótipos onde é encontrado pelo menos um alelo T com a severidade da doença periodontal crônica.

Não foi encontrado o alelo C na posição –1306 do promotor do gene da metaloproteaes-2 (MMP-2) em pacientes normais ou afetados pela doença periodontal crônica na população estudada.

O polimorfismo C/T presente na posição –1562 do promotor do gene da MMP-9 não demonstrou associação com a susceptibilidade ou severidade da doença periodontal crônica.

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ANEXOS

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THE BIOLOGY OF MATRIX METALLOPROTEINASES

BIOLOGIA DAS METALOPROTEASES DA MATRIZ

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Matrix metalloproteinases (MMPs) are an important family of zinc-dependent endopeptidases that mediate the extracellular matrix (ECM) degradation. These enzymes have been implicated in pathologic oral processes such as periodontal tissue destruction, root caries, tumor invasion and temporomadibular joint disorders. In the present work we review some general aspects of matrix metalloproteinases, and discuss the role of theses enzymes in normal physiology and pathology with emphasis on the oral environment.

UNITERMS: Matrix metalloproteinases (MMPs); Oral mucosa.

MATRIX METALLOPROTEINASES: GENERAL ASPECTS

Matrix Metalloproteinases (MMPs) form an important family of metal-dependent endopeptidases that represent the major class of enzymes responsible for degradation of extracellular matrix (ECM) components. Collectively, MMPs are capable of degrading all ECM proteins[2]. All family members are secreted as inactive proenzymes (zymogens) and are thought to be activated in the tissue by cleavage of the propeptide. All MMPs contain Zn^{2+} at the catalytic site and, in addition, require Ca^{2+} for stability and activity.[2]

The first report about a matrix metalloproteinase was published in 1962 by Jerome Gross and Charles Lapière[5]. They found an active enzyme in the culture media of tissue fragments of tail fin skin that degraded the triple helix of native type I collagen. Since then, at least sixteen human MMPs have been characterized (Table 1). MMPs are classified into five main classes (collagenases, gelatinases, stromelysins, membrane-type and others, including the matrilysin) on the basis of their putative substrate specificity and internal homologies.

The members of the MMPs family are organized into three basic, distinctive, and well-conserved domains based on structural considerations: amino-like domain at the carboxy-terminal (Figure 1). The amino acid sequence homology between the MMPs members is highest at the amino-terminal pro-fragment region and the zinc atom catalytic site. Some additional domains or short inserts can be found attached to the common structure in several MMPs, like MMP-2 and –9 that contain a gelatin-binding domain insert between the catalytic and active site domain.[28]

ROLE OF MATRIX METALLOPROTEINASES IN VIVO

Matrix Metalloproteinases are expressed in response to specific stimuli by resident connective tissue cells as well as the major inflammatory cell types that invade the tissue during remodeling events in vivo.[2] Evidences for the role of any particular metalloproteinase in a pathological process is provided by findings as the presence of metalloproteinase mRNA in lesional cells and activity of MMPs in lesions.[9] Such evidences suggest that collagenases could have a fundamental role during ECM degradation since these enzymes have the unique ability to cleave type I collagen that will be further degraded by others proteinases.

The MMPs activity has been related to a number

TABLE 1- The matrix metalloproteinase family.

Enzyme	Number	kDa	Preferred Substrate
Interstitial collagenase	MMP-1	57/52	Helical collagen, proMMP-2, proMMP-9
Neutrophil Collagenase	MMP-8	85-64	Helical collagen
Collagenase-3	MMP-13	52-42	Helical collagen
Gelatinase A	MMP-2	72/66	ProMMP-9, gelatin, fibronectin
Gelatinase B	MMP-9	92/80	Gelatin, fibronectin, elastin, collagen IV, V, VII, X, and denatured type I collagen
Stromelysin-1	MMP-3	60/55	Fibronectin, laminin, elastin, proteoglican, collagen IV, V, IX, X proMMP-1, -7, -8, -9, -13
Stro melysin-2	MMP-10	60/55	Fibronectin, laminin, elastin, proteoglican, collagen IV, V,IX,X
MT1-MMP	MMP-14	66/54	ProMMP-2, -13, helical collagen
MT2-MMP	MMP-15	72/60	----------
MT3-MMP	MMP-16	64/53	ProMMP-2
MT4-MMP	MMP-17	57/53	----------
Matrilysin	MMP-7	28/19	Fibronectin, elastin, collagen IV
Metalloelastase	MMP-12	54/22	Elastin
Enamelysin	MMP-20	54/22	Dental enamel matrix

Fonte: Woessner, 1998

rheumatoid arthritis, osteoarthritis, abdominal aortic aneurysm, acute myocardial infarction and cancer.[29] For tumor cells to metastasize, it produces MMPs in order to break away from its neighbors, force its way into the surrounding stroma, and penetrate the basement membrane until the blood vase. MMPs also participate in normal remodeling processes such as embryonic development, post-partum involution of the uterus, bone remodeling, ovulation and wound healing.[27]

The function of MMPs has been studied using transgenic mice: knockouts for MMP-3, -7, -9 and -12 and over expressions of MMP-1 and -3.[21] The problem of using this kind of analysis appears to be the high functional redundancy of MMPs. The ablation of a particular MMP will induce the expression of other MMPs, in order to compensate for the loss. In view of this fact, it is believed that the multiplicity of MMP forms underlines the extreme importance of these enzymes for the maintenance and repair of the ECM.[28]

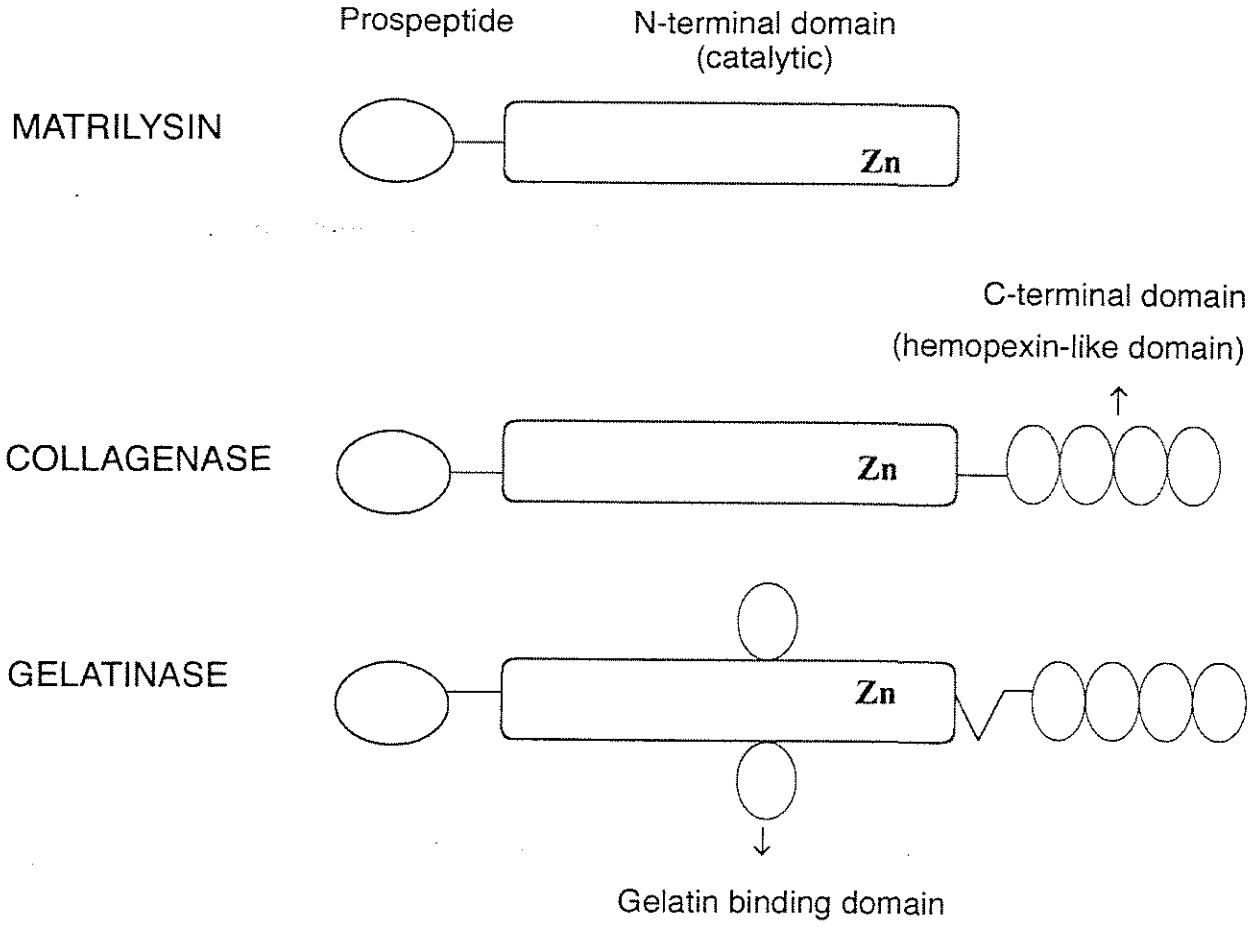

FIGURE 1- Domain Structure Of MMPs.

POLYMORPHISM IN MATRIX METALLOPROTEINASE GENE PROMOTER

The activity of MMPs is regulated at multiple levels, including conversion of proenzyme to the activated form, inhibition by tissue inhibitors of MMPs (TIMPs) in tissues and regulation of transcription. However, the synthesis of MMP appears to represent the key step, since most MMPs genes are expressed only when active physiological or pathological tissues remodeling takes place[29].

Gene promoters are regions that control gene transcription. Recently, DNA polymorphisms have been found in the promoter region of several MMPs. Polymorphism represents natural sequence variants (alleles), which may occur with more than one form, having a frequency greater than 1% in a human population. Approximately, ninety percent of DNA polymorphisms are single nucleotide polymorphisms (SNP) due to single base exchange.

A SNP in the promoter region of human MMP-1 gene has been described.[18] An insertion/deletion of a guanine at position –1607 creates two different alleles, one having a single guanine (1G) and the other having two guanines (2G). The 2G allele together with an adjacent adenosine create a core-binding site (5'-GGA-3') for Ets family of transcription factors that increases the transcriptional activity significantly.[18] Tumors bearing the 2G allele can secrete higher levels of MMP-1, and the presence of this allele was associated with the development of ovarian cancer[8].

Two functionally important genetic polymorphism have been detected in the gene promoter of MMP-9. One of them represents a SNP at position -1562 and the other a (CA)n microsatellite repeat at position -90. The SNP is a C to T substitution that increases the transcriptional activity. Zhang et al.[30] have found an association of the C-1562T polymorphism with the severity of coronary arteriosclerosis. The (CA)n is a multi-allelic microsatellite polymorphism where the most common form has a peak at the $(CA)_{14}$ allele and the second peak at the $(CA)_{21}$, $(CA)_{22}$ and

of the transcriptional activity of MMP-9 promoter comparing with the 21CA repeats. The relationship between the (CA)n repeats and abdominal aortic aneurysm and intracranial aneurysm[14] has been analyzed, but the data are contradictory.

A 5A/6A polymorphism has been reported in the MMP-3 (stromelysin-3) gene promoter. This SNP has been associated to arteriosclerosis in a number of genetic epidemiological studies. The frequency of 5A allele is significantly higher in affected individuals than in control subjects and the risk of acute myocardial infarction in individuals carrying one or two copies of the 5A allele was estimated to be 2.25 fold. MMP-3 is capable to degrade a wide range of extracellular matrix proteins, promoting the cleavage of atherosclerotic plaque.

The evidences presented suggest that genetic polymorphism in the MMPs genes are likely to be related to a wide range of diseases that are characterized by extracellular matrix degradation.

INHIBITORS AND INHIBITION OF MMP ACTIVITY

In the extracellular matrix (ECM), the activity of MMP is controlled by specific inhibitors known as tissue inhibitors of MMPs (TIMPs). TIMPs are small (21-28 kDa), multi-functional proteins that regulate MMP function both at the level of their activation and in their ability to hydrolyze a particular substrate. The MMPs inhibition by TIMPs occurs in a 1:1 stoichiometry and non-covalent fashion.

Four members of TIMP family have been so far described. TIMP-1 is more effective than TIMP-2 at inhibiting MMP-1 and MMP-3. In most cells, MMP-9 is secreted as a complex with TIMP-1, whereas TIMP 2 is associated with MMP-9. Experimental evidences suggest that TIMP-2 is 10 times more effective than TIMP-1 in inhibiting the activity of MMP-2.

The balance between production of MMPs and TIMPs represents a critical point to maintain the homeostasis of the ECM. It is recognized that a pathological breakdown of the ECM can be installed if there is excess of MMP activity in the tissue. For this reason, there is a great interest in the development of synthetic inhibitors of MMP, which could be used in medical therapy. Most attention has been given to zinc chelating agents. Tetracycline was shown to inhibit collagenase in gingival fluid and tissue, independent of their antibacterial activity. Gold salts are used to treat arthritis; these have been shown to work by the binding of a gold atom to a heavy metal site in the MMP distinct from that occupied by the catalytic zinc atom of the MMP[10].

MMPS AND ORAL ENVIRONMENT

Several evidences have supported the fundamental role of MMPs during development and remodeling of oral tissues. MMPs are required to remove the enamel matrix proteins during enamel maturation, resulting in a highly mineralized tissue.[7] MMPs are the major players in collagen breakdown during periodontal tissue destruction.[26] Gingival fibroblasts, keratinocytes, resident macrophages and polymorphonuclear leukocytes (PMN) are capable of expressing MMP-1, MMP-2, MMP-3, MMP-8. MMP-9,[2] inflammatory cytokines and growth factors that up regulate MMPs transcription. High levels of MMPs on the periodontal tissues provoke an imbalance between production and degradation of collagen, causing tooth attachment loss. Periodontitis patients have significantly higher levels of MMP-2 and MMP-9 than health subjects, and the amount of gelatinases decreases after periodontal treatment. The activation of MMP-2 and MMP-9 was also shown to have a crucial role in the destruction of dentin by caries. Additionally, these enzymes can potentate the degradation of extracellular matrix by activating collagenase-3 (MMP-13) and neutrophil collagenase.

Recently, we have demonstrated that divalent metal salts, as Zn, Cu, Hg and Sn, are capable to inhibit the activity of MMP-2 and MMP-9 at low concentration.[24] We have also observed that lead, cadmium and zinc inhibit the activity of enamel matrix proteinases in vitro.[3] Among the metals studied, 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 dentifrices containing zinc salts can reduce plaque accumulation and calculus formation.[4,6,13,15] Using an experimental gingivitis model, Saxton and Cummins[20] have also shown that zinc can effectively improve gingival health. These authors showed that dentifrices containing zinc citrate could reduce the development of gingival inflammation by 25%. Therefore, it is proposed that zinc can directly improve gingival health by directly inhibiting MMPs present in inflamed gingival tissue.

Large amounts of zinc and copper are constantly

released from amalgam[11]. 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[23].

The topical application of zinc oxide has been shown to stimulate the healing of both chronic and acute wounds. Zinc oxide can reduce inflammatory reaction in the granulation tissue and significantly increase re-epithelialization of wounds.[1] The precise functions of MMP-2 and MMP-9 in wound healing are still controversial. The presence of MMP-9 in the leading edge of migrating epithelium suggests that this enzyme may be involved in keratinocyte migration during re-epithelialization after wounding. However, the activities of MMP-2 and MMP-9 were shown to be increased in diabetic healing-impaired mice when compared to nondiabetic mice.[12] The topic application of CMT-2, an inhibitor of MMPs activity, can enhance wound healing in diabetic rats.[16] These data indicate that elevated levels of MMPs may impair wound healing. Zinc oxide is fairly insoluble, it is slowly but continuously dissolved when applied on open wounds.[1] Therefore, zinc oxide can exert a prolonged and constant effect on the healing tissue. The inhibition of MMP-2 and MMP-9 activities may be one mechanism by which topical zinc oxide enhances wound healing[19].

Inhibition of MMPs can be a mechanism to prevent other destructive events. The role of proteolytic enzymes in the root caries process is not totally understood, but there are several evidences indicating that MMPs are required to remove the organic matrix during root-surface caries. Ramamurthy et al.[17] have reported that the treatment with non-antimicrobial tetracycline prevents not only the destruction of periodontium by MMPs, but also avoids the exposure of roots to host tissue. Metalloproteases from peri-implant sulcus fluid have been collected from loosing and well-fixed dental implants. Similar to periodontitis sites, the peri-sulcus fluid of loosing dental implants contains high levels of MMPs.[25] Thus, MMPs inhibitory therapy has order to obtain a better insertion of dental implant to alveolar bone.

RESUMO

As metaloproteases da matriz (MMPs) representam uma importante família de enzimas zinco dependentes que modulam a degradação da matriz extracelular. Estas enzimas têm sido associadas a diversos processos patológicos que afetam a cavidade bucal, como a destruição do tecido periodontal durante a periodontite, cárie de raiz, invasão de tecidos por tumor e desordens da articulação temporomandibular. Neste trabalho nós apresentamos uma revisão dos aspectos gerais das metaloproteases da matriz e discutimos sobre o papel destas enzimas em processos fisiológicos e patológicos, dando ênfase ao papel destas enzimas no meio ambiente bucal.

UINITERMOS: Metaloproteases da matriz (MMPs): Mucosa bucal.

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Gothenburg, February 12, 2002

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Dear Dr. Peres Line,

It is a pleasure for me to inform you that your manuscript "MMP-1 promoter polymorphisms: ...." (CPE1O212b) has been accepted for publication in Journal of Clinical Periodontology. Your material will appear in issue 2 of the year 2003 volume of the journal. Page proofs will be forwarded to you from the printer.

Yours sincerely,

Jan Lindhe

JOURNAL OF Clinical Periodontology

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Dr. Sergio Roberto Peres Line
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Dear Dr. Peres Line,

It is a pleasure for me to inform you that the manuscript by De Souza et al. "Analysis of the TGF-ß$_1$ promoter..." (CPE1O212d) has been accepted for publication in Journal of Clinical Periodontology. Your material will appear in issue 6 of the year 2003 volume of the journal. Page proofs will be forwarded to you from the printer.

Yours sincerely,

Jan Lindhe

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CERTIFICADO

Certificamos que o Projeto de pesquisa intitulado "Análise de polimorfismo na região do promotor dos genes de metaloproteases, TIMPs e TGF-beta: Correlação com a severidade da doença periodontal em adultos e em famílias com periodontite de início precoce", sob o protocolo nº **92/99**, do Pesquisador(a) ***Ana Paula de Souza,*** sob a responsabilidade do Prof(a). Dr(a).Sérgio Roberto Peres Line, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa – FOP.

Piracicaba, 01 de dezembro de 1999

We certify that the research project with title "*Analyze of Gene Promoter Polymorphism of Metalloproteinases, TIMPs and TGF-: Correlation with Periodontal Disease Severity in Adults and Early Onset Periodontitis.*", protocol nº **92/99**, by Researcher ***Ana Paula de Souza***, responsibility by Prof. Dr. ***Sérgio Roberto Peres Line***, is in agreement with the Resolution 196/96 from National Committee of Health/Health Department (BR) and was approved by the Ethical Committee in Research at the Piracicaba Dentistry School/UNICAMP (State University of Campinas).

Piracicaba, SP, Brazil, 01 december 1999

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