PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

FACULDADE DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

LETÍCIA GRANDO MATTUELLA

ANÁLISE DOS POLIMORFISMOS DO GENE HLA-G E DO PADRÃO DE CITOCINAS Th1/Th2 EM PACIENTES COM PERIODONTITE CRÔNICA E AGRESSIVA

Orientador: Prof. Dr. Léder Leal Xavier

PORTO ALEGRE

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Tese apresentada como requisito para obtenção do grau de Doutor pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul.

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DEDICATÓRIA

Dedico esta tese às pessoas fundamentais na minha vida: minha mãe (Vanda), meu pai (Darci), meu marido (Mauro) e minha filha (Fernanda). Obrigada pelo apoio incondicional ao longo destes anos e por acreditarem mais em mim do que eu mesma.

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SUMÁRIO

LISTA DE SIGLAS

AR – artrite reumatóide

bp – *base pairs*

CBA – *cytometric bead array*

Del – deleção

DL – desequilíbrio de ligação

ELISA – ensaio de imunoabsorção ligado à enzima

HLA – antígeno leucocitário humano

HLA-G – antígeno leucocitário humano-G

IFN – interferon

IL – interleucina

Ins – inserção

JIA – artrite idiopática juvenil

MHC – complexo principal de histocompatibilidade

mRNA – RNA mensageiro

NK – *natural killer*

PA – periodontite agressiva

pb – pares de base

PC – periodontite crônica

RT-PCR – reação em cadeia da polimerase em tempo real

Th – T *helper*

TNF – fator de necrose tumoral

UTR – região não traduzida

α – letra grega alfa

γ – letra grega gama

LISTA DE ILUSTRAÇÕES

RESUMO

A periodontite apresenta etiologia bacteriana associada à presença de um hospedeiro suscetível. Fatores imunogenéticos têm sido estudados para tentar explicar as formas mais agressivas da doença, estabelecer um diagnóstico precoce e definir um prognóstico mais confiável. O presente estudo teve como objetivos avaliar os polimorfismos do gene HLA-G (inserção e deleção de 14 pb e C/G +3142) e o perfil de citocinas (Th1 e Th2) em pacientes com periodontites crônica, periodontite agressiva e controles saudáveis. Em relação ao polimorfismo de 14 pb foi observado, nos pacientes com periodontite crônica, um aumento significante na frequência de homozigotos para o alelo de deleção, quando comparados aos controles. Este mesmo grupo apresentou a maior frequência deste alelo, o que foi marginalmente não significante. Além disso, nenhuma diferença significativa foi observada entre os pacientes com periodontite agressiva e os controles em relação aos polimorfismos de 14 pb e C/G +3142. Quando os haplótipos foram estimados, uma frequência aumentada do deleção/G e diminuída do inserção/G foi observada nos pacientes com periodontite crônica comparados aos controles, mas sem diferença estatística. Com relação à concentração sérica de citocinas (IL-2, IL-4, IL-5, IL-10, TNF-α e IFN-γ), não foi verificada diferença significativa entre os grupos estudados, embora os achados revelaram uma tendência a menores níveis de IL-5 e IL-10 no grupo com periodontite agressiva. Nossos resultados sugerem em relação ao HLA-G, que os pacientes homozigotos para o alelo de deleção, têm 3 vezes mais chance de apresentar periodontite crônica (OR = 3.07, 95% CI: 1.24-7.87), inferindo um papel de suscetibilidade deste polimorfismo na patogênese desta condição. Já os pacientes com periodontite agressiva, quando avaliados em relação ao perfil de citocinas, apresentaram uma tendência direcionada ao perfil Th2, sugerindo uma contribuição para o desenvolvimento da manifestação exacerbada da doença.

Palavras-chave: antígeno leucocitário humano-G, polimorfismos, citocinas, periodontite crônica, periodontite agressiva.

ABSTRACT

Periodontitis has a bacterial etiology associated with the presence of a susceptible host. Immunogenetics factors have been studied in an attempt to explain the more aggressive disease, to establish diagnosis and to determine a more reliable prognosis. The present study had as objectives to evaluate the HLA-G polymorphisms (14 bp insertion/deletion and C/G +3142) and the cytokines profile (Th1 and Th2) in patients with chronic periodontitis, aggressive periodontitis and healthy controls. In relation to the 14 bp polymorphism, in chronic periodontitis patients, it was observed a significant increase in homozygous frequency for the deletion allele, when compared to controls. This same group presented a higher frequency of this allele, which was marginally not significant. Furthermore, no significant difference was observed between aggressive periodontitis patients and controls in relationship to the polymorphisms of 14 bp and C/G +3142. When haplotypes were estimated, an increased frequency of the deletion/G and decreased of the insertion/G was observed in chronic periodontitis patients compared to controls, but with no statistical difference. When evaluating serum cytokines concentration (IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ), although no statistical difference could be seen between groups, a tendency to lower levels of IL-5 and IL-10 in aggressive periodontitis group was observed. Our results suggest that having HLA-G homozygosis for the deletion allele, yields three more times chance to present chronic periodontitis (OR = 3.07, 95% CI: 1.24-7.87), inferring a susceptibility role of this polymorphism in the pathogenesis of this condition. Yet considering the cytokine profiles, the aggressive periodontitis patients presented a tendency towards the Th2 profile, suggesting a contribution to the development of this exacerbated manifestation of the disease.

Key-words: human leukocyte antigen-G, polymorphisms, cytokines, chronic periodontitis, aggressive periodontitis.

1 INTRODUÇÃO

1.1 Periodontite

A periodontite é uma condição destrutiva tecidual crônica, na qual, as fibras colágenas do ligamento periodontal que suportam o dente e o osso são danificadas, principalmente, devido ao desenvolvimento de uma resposta imuneinflamatória exacerbada ao biofilme bacteriano (Miranda et al., 2003; Hernández et al., 2011). Bactérias gram-negativas colonizam a área subgengival e são responsáveis pelo início e progressão do processo inflamatório (Firatli et al., 1996). Entretanto, isoladamente, estes micro-organismos são insuficientes para causar a doença, sendo essencial a existência de um hospedeiro suscetível (Offenbacher, 1996; Meng et al., 2007).

Os processos inflamatórios e imunológicos agem nos tecidos periodontais para proteger contra o ataque microbiano. Porém, em alguns indivíduos, essas reações de defesa podem ser prejudiciais, uma vez que, são passíveis de agredir as células e as estruturas vizinhas do tecido conjuntivo. Os critérios estabelecimento, severidade e progressão das periodontites são determinados por fatores ligados à resposta do hospedeiro, além da presença e da virulência bacteriana (Hernández et al., 2011; Page, Kornman, 1997).

Segundo a classificação da Academia Americana de Periodontia (1999), a periodontite crônica (PC) é caracterizada como uma doença de progressão lenta, associada ao acúmulo de biofilme bacteriano, que acomete, geralmente, indivíduos acima de 30/40 anos de idade. Na periodontite agressiva (PA), há rápida destruição do tecido periodontal e ocorre, em sua maior parte, em indivíduos jovens e saudáveis sistemicamente. Em uma amostra representativa de adultos jovens (14 a 29 anos) da região metropolitana de Porto Alegre/Brasil, encontrou-se cerca de 5,5% dos indivíduos com PA, uma prevalência relativamente alta (Susin, Albandar, 2005).

Sabe-se que a periodontite, através da inflamação e da destruição dos tecidos periodontais, produz diversos sinais, sintomas e sequelas clínicas que acabam por impactar, consideravelmente, a qualidade de vida dos indivíduos (Ng, Leung, 2006). O maior impacto é, sem dúvida, a perda dos elementos dentários. Uma observação

importante no estudo de Susin e Albandar (2005) é que o número de dentes perdidos, nos portadores de PA, foi duas vezes maior que em controles sem periodontite, pareados por sexo e idade. Esta forma de periodontite tende a apresentar uma agregação familiar (Stabholz, Soskolne, Shapira, 2010), indicando a importância de fatores genéticos (Shapira et al., 1994; Reichert et al., 2003; Meng et al., 2007). Em alguns casos, a gravidade da doença não demonstra compatibilidade com a quantidade de biofilme bacteriano acumulado, sugerindo uma alta suscetibilidade destes indivíduos à doença (Haas et al., 2006).

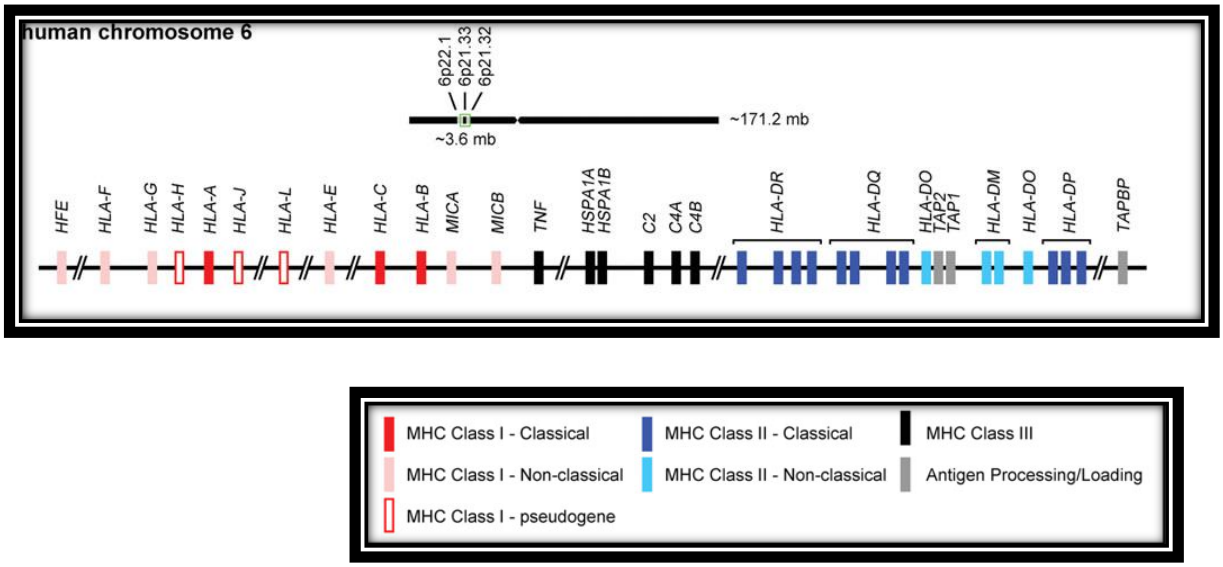
O entendimento dos processos imunológicos que conferem uma maior suscetibilidade às periodontites, de forma especial à PA, devido ao estabelecimento precoce, à rápida destruição tecidual e ao impacto sobre a qualidade de vida, é essencial para o estabelecimento de linhas de ação direcionadas para a prevenção e para melhorias no diagnóstico e no tratamento destas condições. No entanto, as respostas inflamatória e imune envolvidas no estabelecimento e na progressão da destruição tecidual nas periodontites são complexas, tornando este entendimento e a identificação de indivíduos vulneráveis bastante difíceis. Tentativas para tal vêm sendo realizadas, avaliando características morfológicas e funcionais dos tecidos periodontais e do sistema imune inato e adaptativo, bem como polimorfismos de genes associados a condições inflamatórias. A maioria dos genes considerados responsáveis pelo desenvolvimento das periodontites também estão relacionados ao sistema imune. A evidência de uma influência genética em ambas as formas de periodontite existe, mas seu efeito na expressão da doença ainda não é compreendida (Stabholz, Soskolne, Shapira, 2010).

1.2 Complexo principal de histocompatibilidade (MHC)

A coleção de genes localizada no braço curto do cromossomo humano 6 (6p21.3) é conhecida como MHC (Figura 1), ou sistema antígeno leucocitário humano (HLA) (Ohyama et al., 1996). Muitos destes (>10%) desempenham importantes funções na biologia do sistema imune. O HLA desempenha um papel crucial no reconhecimento de antígenos estranhos (Stein et al., 2003). Sabe-se que a resposta imune mediada por células B

(humoral) e T (celular) são iniciadas através de genes contidos dentro do MHC (Rhodes, Trowsdale, 1999). Estudos têm apontado para a contribuição do MHC/HLA como um potente fator genético na etiopatogênese das periodontites (Shapira et al., 1994; Firatli et al., 1996; Machulla et al., 2002; Stein et al., 2008; Repeke et al., 2012).

Figura 1: Mapa genômico do MHC humano (Needleman, McAllister, 2012).



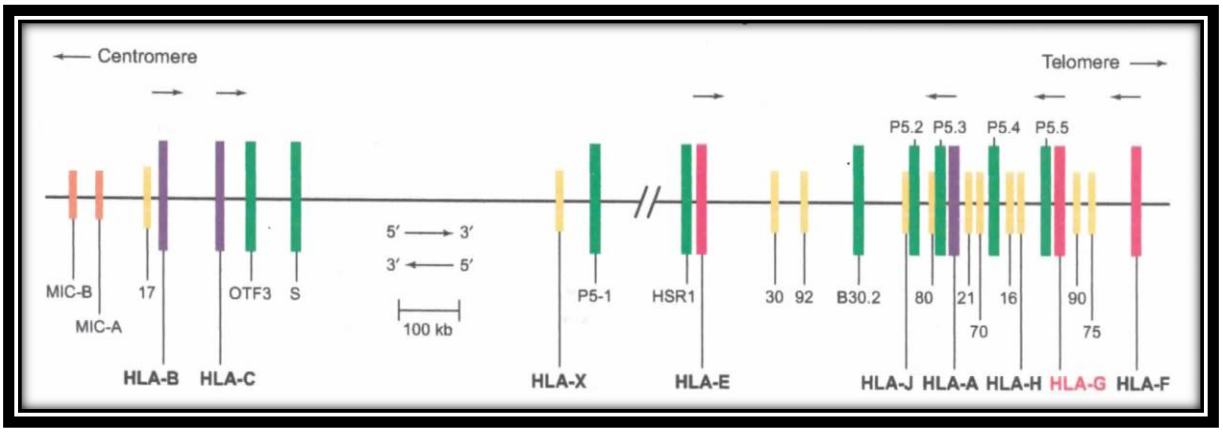
A região do MHC é subdividida em três classes de acordo com as características funcionais de seus genes. As moléculas do MHC de classe I e II têm características estruturais em comum. Ambas atuam no processamento e na apresentação de antígenos e pertencem à família das imunoglobulinas. A região de classe III, situa-se entre as regiões de classe I e II. Seus genes codificam, além de outros produtos, várias proteínas secretadas que apresentam funções imunes, incluindo os componentes do sistema complemento (C2, C4 e fator-beta) e moléculas envolvidas em processos inflamatórios, como o fator de necrose tumoral (TNF) (Kindt, Goldsby, Osborne, 2008). As moléculas de classe II são, funcionalmente, especializadas na apresentação de pequenos fragmentos protéicos (peptídeos antigênicos), principalmente, derivados de proteínas extracelulares, ao receptor de células T nas células T *helper* (Th) CD4+ (Ohyama et al., 1996). Como exemplo, destacam-se os genes HLAs DP, DQ e DR (Rhodes, Trowsdale, 1999).

Os genes da região de classe I (Figura 2) estão envolvidos na apresentação de peptídeos, predominantemente derivados de proteínas intracelulares, às células T citotóxicas (Geraghty et al., 1987; Rhodes, Trowsdale, 1999; Cruvinel et al., 2010). Dentre os classificados como clássicos e pertencentes ao grupo Ia, destaca-se os HLAs A, B e C (Shapira et al., 1994).

Genes clássicos são, aparentemente, duplicados com uma alta frequência no processo evolucionário, e muitos dos genes duplicados parecem se degenerar em genes não-clássicos como resultado de uma mutação deletéria (Hughes, Nei, 1989).

As moléculas de classe I não clássicas (HLAs E, F e G), ou do grupo Ib, tipicamente, perdem três características das moléculas clássicas, sendo elas: a expressão universal, o alto polimorfismo e a função apresentadora de antígeno. Na evolução do *locus* não clássico, tais características não parecem ser perdidas simultaneamente. É esperado que um *locus* não clássico irá, ocasionalmente, reter uma ou mais das características do *locus* clássico (Hughes, Nei, 1989).

Figura 2: Mapa esquemático da região cromossômica HLA de classe I (6p21.3). Genes não clássicos, incluindo o HLA-G, estão evidenciados em rosa (Le Bouteiller, Mallet, 1997).



1.3 Antígeno leucocitário humano-G (HLA-G): polimorfismo de inserção/deleção (ins/del) de 14 pb e C/G +3142

O HLA-G é um gene não clássico de classe I, localizado no braço curto do cromossomo 6, na região 6p21-3 (Hviid et al., 2003; Rizzo et al., 2008). Situa-se na vizinhança do HLA-A e exibe uma estrutura típica de um gene de MHC de classe I clássico, apresentando uma organização semelhante de éxons/íntrons (Le Bouteiller, Mallet, 1997). Sua região promotora contém sequências de DNA conservadas entre as expressas nos genes HLA-A e B (Geraghty et al., 1987).

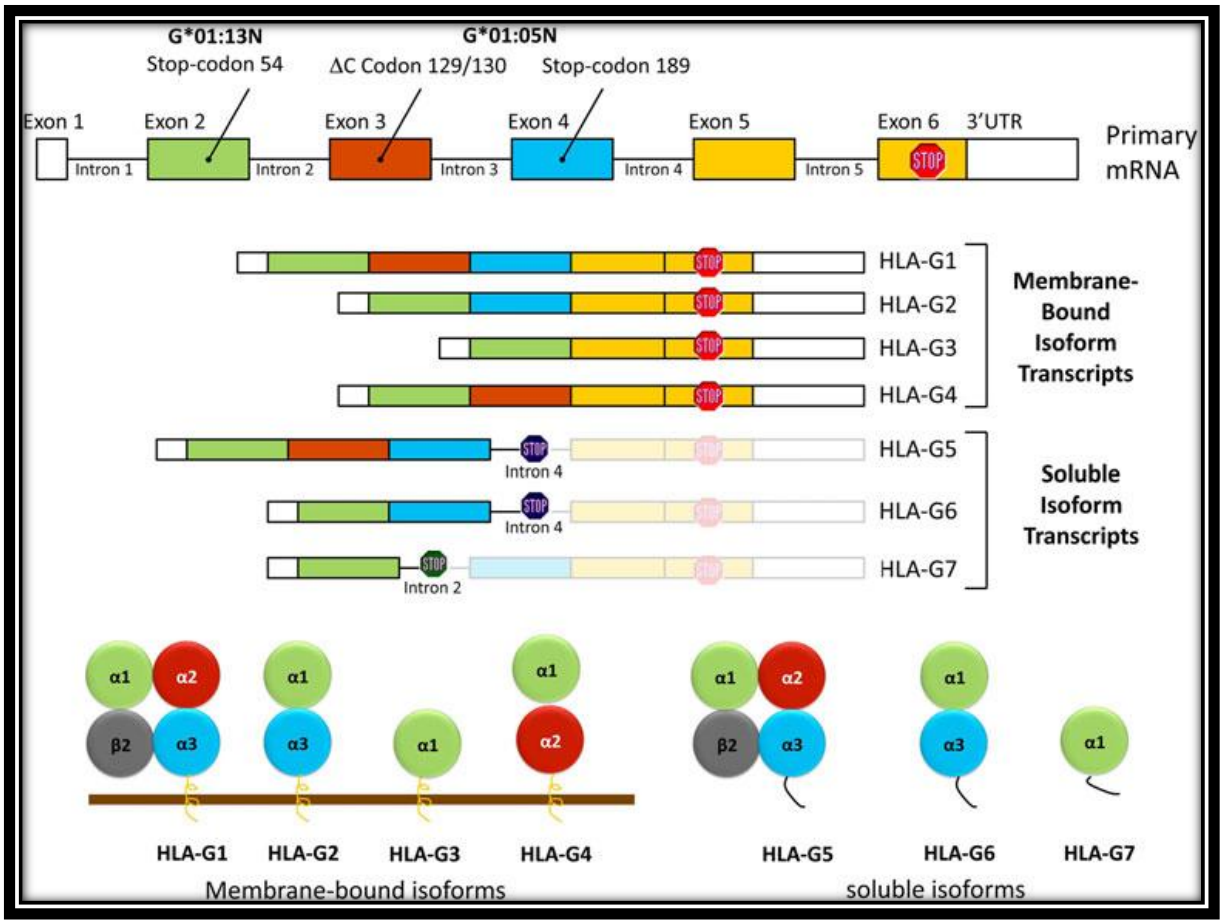
Em contraste com o *locus* do HLA clássico de classe I, o *locus* do HLA-G não clássico tem um polimorfismo limitado (Le Bouteiller, Mallet, 1997; Chen et al., 2008; Cordero et al., 2009), uma restrita distribuição tecidual em condições fisiológicas e apresenta função tolerogênica, ou seja, a célula que o expressa está protegida de destruidores do sistema imune (Carosella et al., 2008). Esta evolução trabalha para evitar mudanças, reduzindo o número de diferentes proteínas de HLA-G, e manter sua função imunológica (Cervera et al., 2010). A molécula de HLA-G interfere em ambas as imunidades, inata e adaptativa (Cordero et al., 2009).

O *locus* HLA-G foi descrito pela primeira vez por Geraghty, Koller e Orr (1987) e a primeira verificação da expressão deste gene foi feita por Kovats et al. (1990) em citotrofoblastos. Sendo assim, esta molécula poderia estar envolvida na interação materno-fetal (Hviid, 2004; Rouas-Freiss et al., 1997; Vianna et al., 2007; Carosella et al., 2008; Carosella, 2011). Ela já foi avaliada, inclusive, no tratamento de fertilização *in vitro* (Jurisicova et al., 1996) e em casos de abortos espontâneos recorrentes (Hviid et al., 2002). O declínio ou aumento do gene HLA-G solúvel no fluido amniótico é capaz de estimular uma resposta imune materna contra o feto e contribuir para o início do parto ou manutenção da gestação, respectivamente (Pistoia et al., 2007; Hviid et al., 2004).

O gene HLA-G é expresso não somente na superfície da membrana das células, mas também nos fluidos corporais na forma de HLA-G solúvel (Pistoia et al., 2007). O transcrito primário do HLA-G produz sete isoformas, quatro das quais ligadas à membrana (G1 a G4) e três solúveis (G5 a G7), (Hviid et al., 2003; Carosella et al., 2008; Cordero et al.,

2009; Carosella, 2011) (Figura 3). Diferenças na expressão do HLA-G podem, parcialmente, serem geneticamente determinadas (Hviid et al., 2004; Veit et al., 2009).

Figura 3: Isoformas do HLA-G produzidas por *splicing* alternativo do RNA mensageiro (mRNA) primário (Donadi et al., 2011).



O principal polimorfismo observado na região 3' não traduzida (3'UTR) do gene HLA-G, na posição 3741, é caracterizado pela presença da ins ou del de 14 pares de base (pb) (rs1704) (Vianna et al., 2007), o que influencia a estabilidade do mRNA (Veit, Chies, 2009). Os transcritos do HLA-G gerados por seus alelos com a sequência de inserção de 14 pb podem estar sujeitos a um *splicing* alternativo adicional, com a remoção de mais 92 pb. Tal acontecimento, confere melhor resistência à degradação do mRNA (Hviid et al., 2003; Rousseau et al., 2003) e altera a função (Cervera et al., 2010) e os níveis da proteína (Hviid et al., 2002; Chen et al., 2008).

O gene HLA-G tem um sítio de ligação para microRNA na 3'UTR, menos de 200 pb distante do sítio polimórfico de 14 pb (Veit, Chies, 2009). Este sítio é um alvo potencial para três microRNAs – miR-148a, 148b e 152. Dentro desta região, situa-se um polimorfismo de um único nucleotídeo C/G, na posição +3142 (rs1063320). O alelo G favorece o alvo destes microRNAs ao sítio de ligação (Tan et al., 2007). É importante salientar que ambos os alelos, apresentam uma frequência, em torno de 50%, em várias populações (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=1063320).

Segundo a hipótese de Veit e Chies (2009), o polimorfismo na posição +3142 é mais diretamente responsável, pela regulação da expressão do HLA-G no nível traducional, que o polimorfismo de 14 pb. Tal fato poderia explicar, por exemplo, a razão pela qual os transcritos originados do alelo +14 pb, embora mais estáveis, não refletem maiores níveis de proteína HLA-G. Sendo assim, não seria válido ter transcritos mais estáveis se eles não são eficientemente traduzidos.

Além do polimorfismo de 14 pb, outros na região promotora do HLA-G, em desequilíbrio de ligação (DL), podem contribuir na expessão do HLA-G solúvel (Chen et al., 2008). Inclusive, já foi demonstrado que os polimorfismos de 14 pb e o C/G +3142, do gene HLA-G, estão em DL (Tan et al., 2007; Consiglio et al., 2011). Uma vez que os antígenos HLA são herdados codominantemente e os alelos do *locus* HLA estão em DL um com o outro, a forma e a força da associação de marcadores individuais deve ser dependente de certas combinações dos antígenos ou haplótipos HLA participantes, respectivamente. Tal fato, representa um fator de variação adicional em termos de potencial associação da doença (Stein et al., 2003).

O HLA-G apresenta efeitos inibitórios, ou seja, propriedades imunossupressivas, através de interações com receptores expressos nas células do sistema imune (Carosella et al., 2001; Carosella et al., 2008). Ele inibe a função citolítica de células *natural killers* (NK) (Le Bouteiller, Mallet, 1997; Rouas-Freiss et al., 1997; Ponte et al., 1999) e linfócitos T, a resposta aloproliferativa de células T CD4+, a proliferação de células T e NK, a maturação de células dendríticas e induz as células T regulatórias (Carosella, 2011).

Em 1997, Le Bouteiller e Mallet sugeriram que pesquisas deveriam ser realizadas para investigar a influência do HLA-G na secreção de citocinas. Alguns anos após, Carosella et al. (2001) concluíram que os efeitos do HLA-G podem ter implicações importantes para o controle do desenvolvimento de condições mediadas por respostas Th1/Th2, incluindo a manutenção da gestação (Kanai et al., 2001). Sua expressão regula o balanço entre estas células e promove a polarização Th2. A interleucina (IL)-10, secretada durante as fases iniciais da resposta imune, induz a expressão do HLA-G, ligado à membrana e solúvel, por macrófagos e monócitos ativados (Moreau et al., 1999). Ainda, o HLA-G diminui a secreção e IFN-γ e fator de necrose tumoral-α (TNF-α), mas aumenta a liberação de IL-3, IL-4 e IL-10 pelas células Th (Carosella et al., 2001). Resultados semelhantes foram encontrados por Kanai et al. (2001), entretanto, em relação à IL-10 não foi observada modificação na quantidade de sua expressão, em células cultivadas com HLA-G na forma ligada à membrana. A habilidade do HLA-G em ser sobre regulado pela IL-10 poderia representar um papel na regulação de respostas imunes durante os processos inflamatórios (Moreau et al., 1999).

Condições inflamatórias podem estar associadas com a expressão do HLA-G (Moreau et al., 1999). O polimorfismo deste gene tem sido estudado, por exemplo, em pacientes com: lúpus eritematoso sistêmico (Rizzo et al., 2008; Veit et al., 2009; Consiglio et al., 2011), artrite idiopática juvenil (JIA) (Veit et al., 2008) artrite reumatóide (AR) (Veit et al., 2008), anemia falciforme (Cordero et al., 2009), esclerose múltipla (Wiśniewski et al., 2010) e doença arterial coronariana (Boiocchi et al., 2012).

1.4 HLA e periodontite

Pacientes com AR e periodontite apresentam várias semelhanças, como a presença de uma resposta inflamatória persistente, as respostas humoral e celular ocorrendo em áreas especializadas compostas por tecido conjuntivo e ósseo, a ativação do sistema complemento, a produção de citocinas e a liberação de outros produtos inflamatórios que resultam na destruição dos tecidos supra citados (Firatli et al., 1996). Reichert et al. (2007)

avaliaram antígenos HLA em comum, na JIA e na periodontite e concluíram que o alelo HLA-DRB3 pode ser um indicador de risco em comum, entre pacientes do sexo feminino, com JIA e PC. Além disso, assumiram que uma resposta imune dependente de HLA a bactérias periodontopatogênicas poderia influenciar o curso de ambas as doenças.

A suscetibilidade ou resistência à PC e à PA podem ser influenciadas por combinações de marcadores de HLA. Com relação aos seus polimorfismos, observa-se diferenças inter-individuais na resposta imune contra antígenos bacterianos (Stein et al., 2008). Alguns grupos de pesquisa já estudaram as seguintes moléculas HLA em pacientes, de diferentes etnias, com periodontite: HLA-A, B, C e DR (Shapira et al., 1994; Firatli et al., 1996), HLA-DP e HLA-DR (Ohyama et al., 1996), HLA-DQ (Hodge, Riggio, Kinane, 1999), HLA-A, B, C, DR e DQ (Machulla et al., 2002; Reichert et al., 2003; Stein et al., 2003; Reichert et al., 2007) HLA-B e HLA-DR (Repeke et al., 2012). Entretanto, até o presente momento, nenhum estudo analisou o HLA-G e seus polimorfimos em pacientes com este perfil de doença. O papel de um mecanismo biológico associando o HLA e as doenças periodontais permanece evasivo segundo Stabholz, Soskolne e Shapira (2010).

Pelo fato da molécula HLA-G estar envolvida em vários processos imunoregulatórios e em desordens inflamatórias, ela representa um excelente gene candidato para associação com a periodontite.

1.5 Perfil de citocinas e periodontite

Citocinas são pequenas proteínas solúveis que conduzem informações de uma célula para outra (Callard, George, Stark, 1999). Seu efeito final é regulado em nível de expressão gênica, transcrição, secreção protéica e interação com células-alvo através da ligação com o receptor e, posterior, sinalização celular (Bendtzen, 1994).

O resultado de um processo infeccioso pode ser atribuído ao balanço relativo entre estas moléculas. Uma definição funcional de citocinas diferencia entre os tipos Th1 e Th2. As primeiras, tendo como o TNF-α e IFN-γ seus principais representantes, estão

envolvidas na ativação de células T citotóxicas e macrófagos, consequentemente, estimulam a imunidade celular e a inflamação (Hernández et al., 2011). As segundas (IL-4, IL-5 e IL-10) promovem a imunidade humoral, contra balanceando a resposta Th1 e, desta forma, agindo como moléculas anti-inflamatórias (Romagnani, 1997; Belardelli, Ferrantini, 2002). Citocinas Th1 e Th2 desempenham um papel crucial nas respostas imune e inflamatória, e o resultado de uma infecção pode ser atribuído ao balanço na razão relativa entre elas (Górska et al., 2003).

O TNF-α é uma potente citocina pró-inflamatória, capaz de induzir a destruição tecidual e reabsorção óssea. Já a IL-4 é considerada uma citocina anti-inflamatória, por possuir funções importantes na modulação das células B e na regulação dos macrófagos. Alterações no seu gene podem aumentar a severidade da doença através de uma sub regulação na produção de IL-4. A IL-10 também é uma citocina anti-inflamatória, a qual modula a expressão da doença na PC. Polimorfirmos no seu gene parecem influenciar a regulação de sua expressão (Meng et al., 2007).

O conceito de periodontite, como previamente explorado, envolve uma condição complexa. Análise do perfil de citocinas (Th1 e Th2) em tecidos periodontais afetados pela doença na sua forma crônica já foi realizado utilizando estratégias distintas, como a hibridização *in situ* e a imunoistoquímica (Lappin et al., 2001), o ensaio de imunoabsorção ligado à enzima (ELISA) (Górska et al., 2003; Havemose-Poulsen et al., 2005; Duarte et al., 2010), a reação em cadeia da polimerase em tempo real (RT-PCR) (Garlet et al., 2003; Suárez et al., 2004) e, mais recentemente, a técnica de *cytometric bead array* (CBA) (Queiroz et al., 2008; Andrukhov et al., 2011; Mattuella et al., 2012). Esta última, permite a quantificação simultânea de proteínas por citometria de fluxo e, segundo o fabricante, apresenta vantagens em relação à ELISA, como a obtenção de uma curva padrão para cada analito estudado a partir de um único conjunto de padrões diluídos. Ainda, permite mensurar, simultaneamente, seis citocinas pertencentes aos perfis Th1 e Th2, minimizando erros metodológicos.

Recentemente, uma nova linhagem de células T, capazes de produzir IL-17 e conhecidas, portanto, como Th17, tem sido descrita. A IL-17 age sobre os osteoblastos, aumentando a expressão de RANKL, induzindo, diretamente, a diferenciação de progenitores de osteoclastos em osteoclastos maduros e é capaz de estimular a produção de proteína C-reativa (Bi, Liu, Yang, 2007; Kramer, Gaffen, 2007). Segundo Hernández et al. (2011), as células Th17 representam o fenótipo Th osteoclastogênico envolvido na periodontite progressiva.

Alguns resultados controversos foram encontrados nos estudos acima citados. Por exemplo, níveis elevados de IL-10 (Lappin et al., 2001; Havemose-Poulsen et al., 2005; Andrukhov et al., 2011) e diminuídos de IL-2 (Lappin et al., 2001; Andrukhov et al., 2011) e IFN-γ (Lappin et al., 2001) foram observados em pacientes com periodontite comparados a controles saudáveis. A IL-4 estava significativamente presente na PA (Lappin et al., 2001), mas não na PC (Górska et al., 2003). Queiroz et al. (2008) utilizaram a técnica de CBA para avaliar os níveis de IL-2, IL-4, IL-5, IL-10, TNF-α e IFN-γ e não observaram diferença estatística no perfil de citocinas em pacientes com PC.

2 JUSTIFICATIVA

A análise de parâmetros genéticos e imunológicos de indivíduos com periodontite é essencial para o estabelecimento de linhas de ação direcionadas para a prevenção desta condição patológica. Apesar da baixa prevalência da PA na população em geral, o estudo desta doença e a comparação com dados provenientes de pacientes com PC poderão revelar variantes genéticas, potencialmente, envolvidas no desenvolvimento de doenças inflamatórias crônicas. Assim, considerando o envolvimento de fatores genéticos ainda não exatamente determinados e a importância do sistema imune na suscetibilidade à periodontite, tanto crônica quanto agressiva, estamos propondo a caracterização de pacientes através de uma abordagem imunogenética que se desenvolve tanto pela análise das variantes polimórficas do gene HLA-G quanto pela análise do padrão de citocinas.

3 OBJETIVOS

3.1 Objetivo geral

Analisar o perfil imunogenético de pacientes com periodontite.

3.2 Objetivos específicos

Determinar as frequências alélicas e genotípicas do polimorfismo de ins/del de 14 pb na região 3'UTR do gene HLA-G em pacientes com periodontite *versus* saudáveis.

Determinar as frequências alélicas e genotípicas do polimorfismo C/G +3142 na região 3'UTR do gene HLA-G em pacientes com periodontite *versus* saudáveis.

Determinar a frequência dos haplótipos do polimorfismo de ins/del de 14 pb e do polimorfismo C/G +3142 na região 3'UTR do gene HLA-G em pacientes com periodontite *versus* saudáveis.

Avaliar os níveis séricos de citocinas Th1/Th2 em pacientes com periodontite *versus* saudáveis.

4 ARTIGOS CIENTÍFICOS

4.1 Artigo submetido para publicação no periódico Clinical Oral Investigations (Fator de impacto = 2.364)

Title Page

Letícia Grando Mattuella[1], Lisiane Bernardi[2], Francis Maria Báo Zambra[3], Milene Borges Campagnaro[1], Rui Vicente Oppermann[4], Léder Leal Xavier[1], José Artur Bogo Chies[3], Letícia Algarves Miranda[1]

HLA-G gene polymorphisms in chronic and aggressive periodontitis: a preliminary study

1 Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Laboratório de Biologia Tecidual, Porto Alegre/Brasil.

2 Universidade Federal do Rio Grande do Sul (UFRGS), Laboratório de Biologia Bucal, Porto Alegre/Brasil.

3 Universidade Federal do Rio Grande do Sul (UFRGS), Laboratório de Imunogenética, Porto Alegre/Brasil.

4 Universidade Federal do Rio Grande do Sul (UFRGS), Departamento de Odontologia Conservadora – Periodontia, Porto Alegre/Brasil.

Corresponding author:

Letícia Grando Mattuella

Av. Ipiranga 6681, Bloco 12A, Sala 144, Porto Alegre, RS/Brasil

90619-900

Tel: + 55 51 3320 3545

Email: lmattuella@gmail.com

Abstract

Objective: The present study had as objective to evaluate the HLA-G polymorphisms in patients with periodontitis and healthy controls. **Materials and methods:** The insertion/deletion polymorphism of 14 base pairs and a single nucleotide polymorphism C>G on the position +3142 of the 3' untranslated region of the gene were analyzed in chronic periodontitis (n=62), aggressive periodontitis (n=24) patients and healthy control (n=47) using PCR technique. **Results:** In relation to the 14 base pairs polymorphism, in chronic periodontitis patients, it was observed a significant increase in homozygous frequency for the deletion allele, when compared to controls. This same group presented a higher frequency of this allele, which was marginally not significant. Furthermore, no significant difference was observed between aggressive periodontitis patients and controls in relationship to the polymorphisms of 14 base pairs and C/G +3142. When haplotypes were estimated, an increased frequency of the deletion/G and decreased of the insertion/G was observed in chronic periodontitis patients compared to controls, but with no statistical difference. **Conclusion:** Our results suggest that having HLA-G homozygosis for the deletion allele, yields three more times chance to present chronic periodontitis (OR = 3.07, 95% CI: 1.24-7.87), inferring a susceptibility role of this polymorphism in the pathogenesis of this condition. **Clinical relevance:** This study shows for the first time that patients homozygotes for the 14 base pair deletion allele of the HLA gene are three times more likely to develop chronic periodontitis, suggesting a susceptibility role of this polymorphism in the pathogenesis of this condition.

Key-words: human leukocyte antigen-G, polymorphisms, chronic periodontitis, aggressive periodontitis.

Introduction

Periodontitis is a chronic tissue destructive condition in which the tooth supporting collagen fibers of the periodontal ligament and bone are broken down mainly due to the development of an exacerbated immune inflammatory response to the dental biofilm [1]. According to the American Academy of Periodontology [2], chronic periodontitis (CP) is classified as a slow progression disease related to an accumulation of bacteria biofilm. This form of the disease usually manifests in people over 30/40 years old. Aggressive periodontitis (AP) is characterized by a rapid periodontal destruction, generally occurring in young patients [3], and usually the severity of the disease is not compatible with the amount of biofilm. The inter-individual differences in the outcome and course of periodontitis are not explained only by microbial factors. It is known that only a limited group of individuals develop AP while others have no or only slowly disease progression (chronic) [4].

Human leukocyte antigen (HLA) gene polymorphisms can provide important susceptibility or resistance factors for periodontitis [4]. The HLA system, if implicated in periodontitis, may be associated with low or non-responsiveness to bacterial antigen, which could result in disease progression [5]. HLA-G is a class I non-classical major histocompatibility complex (MHC) molecule characterized by restricted expression and low DNA polymorphism. It has limited tissue distribution under normal physiological conditions and by alternative splicing can produce seven different isoforms, four membrane-bound (HLA-G1 to -G4) and three soluble (HLA-G5 to -G7) [6,7].

As an HLA molecule, the HLA-G shares structural properties of its classical counter-parts HLA-A, B and C [6]. On the 3' untranslated region (UTR) of the HLA-G gene an insertion/deletion (ins/del) polymorphism of 14 base pair (bp) (rs1704) influences the mRNA stability [8], play a role in alternative splicing and is associated with different levels of HLA-G in plasma [9]. Also, in this region, there is a single nucleotide polymorphism (SNP) C>G on the position +3142 (rs1063320) which is known to be within a putative binding site for microRNAs, which is thought to be relevant for the regulation of the HLA-G expression [6].

In sites of inflammation and in inflammatory diseases, such as juvenile idiopathic arthritis (JIA) [10], rheumatoid arthritis (RA) [10], systemic lupus erythematous (SLE) [11,12], multiple sclerosis [13], angiographic coronary artery disease [14], HLA-G gene was shown to be expressed. This molecule is involved in a possible mechanism of tissue protection against inflammatory response [15] and it is able to mediate the inhibition of the cytotoxic activity of natural killer and CD8 T-cells, affect CD4 T-cell functions and dendritic cell maturation [7].

Until now, several HLA gene polymorphisms have been investigated as possible markers of susceptibility to periodontitis but studies vary in inclusion criteria, methodological procedures and results [16-20]. Concerning HLA-G, its relation to CP and AP has not been described so far. Since HLA-G molecule seems to be involved in several other inflammatory disorders as mentioned above, the aim of the present study was to assess the influence of two polymorphisms located at the 3'UTR of the HLA-G gene in patients with CP and AP.

Materials and methods

Patients and controls

One hundred and thirty three patients were selected from the Department of Periodontology at the Federal University of Rio Grande do Sul (UFRGS), Brazil. Their age ranged from 12 to 64 years. After clinical examination, the patients who fulfilled the clinical inclusion criteria (see below) were invited to participate in the present study, which was submitted to and approved by the UFRGS (protocol no.: 47/05) and PUCRS (protocol no.: 272/08) Ethics Committees. All patients and individuals from the control group were thoroughly informed about the purpose and methods of the study and a written consent was obtained from each of them. Subjects were classified into the following groups: healthy control (n=47), CP (n=62) and AP (n=24). Control healthy subjects had at least 20 teeth in the mouth, gingival bleeding index less than 20%, attachment loss and/or probing depth in proximal sites $\leq$ 3 mm. All periodontal examinations were performed by post-graduated periodontologists. CP subjects were aged between 35 and 60 years and had at least 20 teeth, attachment loss $\geq$ 4 mm in at least 10 teeth, probing depth $\geq$ 6 mm in at least 5 teeth and

periodontal bleeding in at least 10 teeth [21]. AP subjects were those who had four or more teeth with attachment loss ≥ 4 mm in individuals between 14 and 19 years old or ≥ 5 mm in individuals between 20 and 29 years old [3]. A questionnaire was applied to all participants regarding their educational level, smoking habits and general health. For the present research, individuals diagnosed with HIV and/or diabetes, pregnant or lactiferous women and anyone who took immunomodulatory/anti-inflammatory/antibiotics drugs in the 6 months prior to the study were excluded.

Genotyping of the HLA-G gene polymorphism

Blood samples (15 mL) were obtained by venipuncture from each patient using BD™ Vacutainer tubes (BD Diagnostics, NJ, USA) and DNA was extracted with a salting-out method [22]. Genotyping was performed by PCR amplification using specific primers. For the genotyping of the +3142 C>G polymorphism, 200 ng genomic DNA were prepared to a final volume of 25 µl [23], with final concentrations as follows: 10 pmol of each primer (DIRECT: 5'-CATGCTGAACTGCATTCCTTCC-3' and REVERSE: 5'-CTGGTGGGACAAGGTTCTACTG-3'), 0.2 mM of each dNTP, 2.0 mM $MgCl_2$, PCR buffer 1X and 1.0 U of Taq DNA polymerase (Invitrogen Corporation, CA, USA). Samples were submitted to 94°C for 5 min, 32 cycles of 30 s at 94°C, 30 s at 65.5°C and 60 s at 72°C followed by a final extension step of 5 min at 72°C. The PCR products were digested with 3 U of the restriction enzyme BaeGI (New England Biolabs Inc., MA, USA), according to manufacturer's instructions, producing 316 and 90 bp fragments for the G allele or a single undigested 406 bp fragment for C allele, which were visualized under UV irradiation in a 2% agarose gel stained with ethidium bromide. For the genotyping of the 14 bp polymorphism of the HLA-G gene, samples were amplified in a 25 µl reaction, according to Hviid et al. [24] with final concentration as follows: 100 ng genomic DNA, 10 pmol of each primer, dNTP 0.2 mM, $MgCl_2$ 1.5 mM, PCR buffer 1X and Taq DNA polymerase 0.75 U (Invitrogen Corporation, CA, USA). Samples were submitted to 2 min at 94°C, 35 cycles of 30 s at 94°C, 60 s at 64°C and 60 s at 72°C followed by a final extension of 10 min at 72°C. The set of specific primers used in these amplification reactions were Primer A: 5'-GTGATGGGCTGTTTAAAGTGTCACC-3' and Primer B: 5'-GGAAGGAATGCAGTTCAGCATGA-3'. PCR products were plotted on 8%

polyacrylamide gel stained with ethydium bromide and visualized under UV irradiation. The 14 bp ins allele amplification yielded a 224 bp fragment whereas the del allele amplification yielded a fragment of 210 bp.

Statistical analysis

The 14 bp and +3142 C>G genotypic frequencies were compared to Hardy-Weinberg (HW) expectations using Chi-squared tests. The allelic and genotypic frequencies of the HLA-G gene polymorphisms of periodontitis patients and controls were compared using the Fisher's exact test or Chi-squared test (with Yates correction when necessary). Haplotype frequencies were estimated with the MLocus software [25], which uses an expectation maximization algorithm [26]. Relative risks were estimated by the odds ratio. The significance level was set at $\alpha = 0.05$ (two-tailed), and all statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, Illinois, USA) and winPEPI [27].

Results

General characteristics of the subjects included in the study are presented in Table 1.

Patients and control genetic data are displayed on Table 2. Considering the 14 bp ins/del polymorphism, we observed a significant deviation from HW expectations in the CP group ($p = 0.047$), but not in the healthy control and AP groups, due to a deficit in heterozygosis. No significant deviations were observed in patients and control groups considering the +3142 C>G SNP.

A significantly increased frequency of homozygotes for the 14 bp del allele was observed in the CP group as compared to controls (0.484 against 0.234, OR = 3.07, 95% CI: 1.24-7.87). The CP group presented a higher frequency of the del allele, which was marginally not significant (0.653 against 0.533, $p = 0.053$). No significant differences were observed between the AP and healthy control groups concerning the genotypic and allelic frequencies of this polymorphism. Also, no significant differences were observed among patients and controls when considering the +3142 C>G SNP frequencies.

Considering the estimated haplotype frequencies from both polymorphisms, we observed an increased frequency of the del/G haplotype in CP patients (0.177 against 0.085) and, conversely, a decreased frequency of the ins/G haplotype (0.346 against 0.478). However, these differences did not reach statistical significance ($p = 0.057$).

Discussion

This study assessed the influence of two polymorphisms of the HLA-G gene in patients with CP and AP. These polymorphisms have been studied in several other tissue-destructive inflammatory diseases [10-14] but this is the first study for periodontitis. Considering the immune-inflammatory tissue-destructive nature of periodontitis, a role for a molecule involved in several immunoregulatory processes, such as the HLA-G molecule, seems suitable to be investigated in the etiopathogenesis of these diseases.

We observed a significant increased frequency of homozygotes for the HLA-G 14 bp del allele for the CP patients compared to controls, showing an OR of 3.07 (95% CI: 1.24-7.87). Although no statistical significance was observed in the HLA-G -14 bp allele, this polymorphism was more frequent in the patients groups (CP and AP). Interestingly, Veit et al. [10] also found that the HLA-G -14 bp allele was a risk factor for another chronic tissue destructive condition, JIA.

A biological explanation for this finding could involve the anti-inflammatory cytokine IL-10. Rizzo et al. [28] performed an in vitro study using peripheral blood mononuclear cells activated with lipopolysaccharide and observed a relationship between +14 bp/+14 bp cells and higher IL-10 levels. Considering IL-10 as an anti-inflammatory cytokine, the -14 bp allele could be associated to lower IL-10 levels in vivo and in inflammation situations, such as periodontitis. Indeed, in periodontitis patients, an absence of IL-5 and IL-10 expression was observed in gingival biopsies [29]. Górska et al. [30] compared severe CP patients with healthy controls and analyzed cytokine concentration in inflamed gingival tissue and serum samples. They found that although IL-10 levels were generally low or even undetectable in serum, the frequency of individuals expressing IL-10 positive cells was much higher in healthy gingival tissue as compared to CP patients. These findings are corroborated by Garlet et al. [31] who observed lower IL-10 expression in

gingival biopsies from patients with AP. We also observed a trend towards lower serum levels of Th2 cytokines, including IL-5 and IL-10, in AP patients [32]. However, additional studies should specifically correlate IL-10 levels with HLA-G -14 pb polymorphism in periodontitis patients to clarify this issue. We have not observed any of the polymorphisms analyzed to be susceptibility indicators for the aggressive form of periodontitis. This may be due to the reduced sample size of this group of patients presenting this more rare form of the disease. The present study could, thus, be considered a preliminary report and additional studies with a bigger sample could render more reliable results.

We had no haplotype ins/C in our study. However, del/C was more frequent, but not statistically significant, in the CP group. In the control and AP group, very similar results were found to ins/G and del/C. Different findings from this were demonstrated in lupus patients [33] and C hepatitis patients [23], where the haplotype del/C was suggested to be a susceptibility factor for these diseases.

An interesting meta-analysis has not observed a positive nor negative significant association between HLA gene polymorphisms and patients with chronic periodontitis. However, in aggressive patients, a positive significant association was described to HLA-A9 and HLA-B15 polymorphisms, while, HLA-A2 and HLA-B5 showed a negative association. It is important to mention that all studies included had Caucasian patients in their sample [4]. These same authors believe that differences between the prevalence of certain bacteria in the subgingival biofilm in different countries and in different ethnic groups may influence the results.

Racial genetic variations could also influence HLA-G gene polymorphisms. In the study of Lucena-Silva et al. [34], the majority of polymorphic sites showed a similar distribution in healthy individuals and the genetic background of Northeastern and Southeastern Brazilian populations could influence the HLA-G gene variability. Even though these findings are not be related to the presence of a chronic inflammation like ours, it was observed a similarity in the allele and haplotypes frequencies for the 14 pb del/ins and +3142 C>G polymorphisms of the HLA-G gene when their results were compared to ours control group. This could be justified by the fact that the population of the present study is also originated from the South of Brazil, as the mentioned study.

Concluding, no differences were found among patients (CP and AG) and controls when considering the +3142 C>G SNP and haplotypes frequencies, but a significant increased frequency of homozygotes for the 14 bp del allele was observed in CP patients compared to healthy controls, suggesting a susceptibility role of this polymorphism in the pathogenesis of this condition.

Acknowledgments

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Table

Table 1. Characteristics of the studied subjects.

	Healthy group	Periodontitis group	
		Chronic	Aggressive
Male/Female gender (%)	14/ 33 (29.8/ 70.2)	23/ 39 (37.1/ 62.9)	04/20 (16.7/ 83.3)
Age (mean and SD)	29.7 (±0.7)	48.1 (±7.8)	34.9 (±.9.8)
Ethnic group (%)			
Caucasian	47 (100)	52 (83.9)	20 (83.3)
Non Caucasian	0 (0)	10 (16.1)	04 (16.7)
Smoking status (%)			
Non smoker	34 (72.3)	25 (40.3)	15 (62.5)
Former smoker	06 (12.8)	22 (35.5)	03 (12.5)
Smoker	07 (14.9)	15 (24.2)	06 (25)

Table

Table 2. HLA-G genotypic and allelic frequencies in patients and controls.

	Controls *Frequency (N)*	*Chronic Periodontitis* *Frequency (N)*	*Aggressive Periodontitis* *Frequency (N)*
14 bp			
Del/del	0.234 (11)	0.484 (30)	0.375 (9)
Ins/del	0.574 (27)	0.339 (21)	0.375 (9)
Ins/ins	0.191 (9)	0.177 (11)	0.250 (6)
OR (95%CI)[a]	-	**3.07 (1.24-7.87)**[b]	1.96 (0.58-6.46)
Del	0.533 (49)	0.653 (81)	0.562 (27)
Ins	0.467 (45)	0.347 (43)	0.438 (21)
P-value	-	0.053	0.723
+3142			
CC	0.128 (6)	0.274 (17)	0.250 (6)
CG	0.617 (29)	0.403 (25)	0.375 (9)
GG	0.255 (12)	0.323 (20)	0.375 (9)
OR (95%CI)[c]	-	2.58 (0.86-8.72)	2.28 (0.52-9.73)
C	0.436 (41)	0.476 (59)	0.438 (21)
G	0.564 (53)	0.524 (65)	0.562 (27)
P-value	-	0.585	1.000
Haplotype[d]			

del/C	0.436 (41)	0.475 (59)	0.437 (21)
del/G	0.085 (8)	0.177 (22)	0.125 (6)
ins/G	0.478 (45)	0.346 (43)	0.437 (21)
P-value	-	0.057	0.732

[a] Taking del/del as reference genotype. [b] P_{corr}=0.020. [c] Taking CC as reference genotype. [d] Estimated frequencies.

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ORIGINAL ARTICLE

Plasma cytokines levels in aggressive and chronic periodontitis

LETÍCIA GRANDO MATTUELLA[1], MILENE BORGES CAMPAGNARO[1], ANDRÉIA ESCOSTEGUY VARGAS[2], LÉDER LEAL XAVIER[1], RUI VICENTE OPPERMANN[3], JOSÉ ARTUR BOGO CHIES[2] & LETÍCIA ALGARVES MIRANDA[1]

[1]*Laboratório de Biologia Tecidual, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, Brazil,* [2]*Laboratório de Imunogenética, and* [3]*Departamento de Odontologia Conservadora – Periodontia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil*

Abstract

Objective. The present study evaluated the Th1/Th2 cytokine profile in plasma from healthy controls and different types of periodontitis patients. ***Materials and methods.*** The concentration of IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ was determined in healthy controls ($n = 18$) and patients with chronic ($n = 19$) and aggressive periodontitis ($n = 19$) using a flow cytometric multiplex immunoassay. Means and standard deviations were calculated and compared using Kruskal-Wallis test. Spearman rho coefficient was used to correlate cytokines in the studied groups. ***Results.*** Although there was no significant difference in the concentration of cytokines between groups, there was a tendency to lower levels of IL-5 and IL-10 in the aggressive periodontitis group. Stronger correlations were observed between IL-2/IL-4 and IL-2/IL-10 in healthy controls (0.938 and 0.669, respectively) compared with chronic (0.746 and 0.532) and aggressive periodontitis groups (0.395 and 0.266). When compared to healthy (0.812) and chronic periodontitis (0.845) groups, the correlation of IL-4/IL-5 was weaker in the aggressive group (0.459). ***Conclusion.*** No difference between systemic levels of Th1/Th2 was observed. In aggressive periodontitis patients, nevertheless, a trend towards low levels of Th2 cytokines could suggest a contribution to the development of such an exacerbated manifestation of this disease.

Key Words: *periodontitis, cytokines, plasma, flow cytometry multiplex assay*

Introduction

Periodontitis is a chronic tissue destructive condition in which the tooth supporting collagen fibers of the periodontal ligament and bone are broken down, mainly due to the development of an exacerbated immune inflammatory response to the dental biofilm [1]. The local ecological conditions of the gingival sulcus and host response can influence the opportunistically overgrow of periodontal bacteria [2] and be able to promote destructive periodontitis resulting in irreversible loss of connective tissue and bony attachment [3]. According to the American Academy of Periodontology [4], chronic periodontitis is classified as a slow progression disease related to an accumulation of bacteria biofilm. This form of the disease usually occurs in people over 30/40 years old. Aggressive periodontitis is characterized by a rapid periodontal destruction and generally occurs in young patients with no concomitant systemic diseases. Familial aggregation can occur and sometimes the severity of the disease is not compatible with the amount of biofilm, suggesting a high susceptibility to tissue destruction. As a result, these susceptible patients are twice as likely to lose their teeth when compared to healthy subjects matched by age and sex [5]. Immunological features, such as cytokine profiles, have been studied in an attempt to better understand the behavior of the immune system in these highly susceptible patients [6,7,9,10,12,13].

Cytokines, small soluble proteins that convey information from one cell to another [14], play essential roles in immune and inflammatory responses and the outcome of infection may be attributable to the

Correspondence: Letícia Algarves Miranda, Av. Ipiranga 6681, Bloco 12A, Sala 144, Porto Alegre, RS, 90619-900, Brazil. Tel: + 55 51 3320 3545. E-mail: leticia.miranda@pucrs.br

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relative balance between such molecules. A functional definition of cytokines distinguishes between Th1 and Th2 cytokines. Briefly, Th1 cytokines (with TNF-α and IFN-γ as typical representatives) are involved in the activation of T cytotoxic cells and macrophages, consequently stimulating cellular immunity and inflammation, whereas Th2 cytokines (IL-4, IL-5 and IL-10) promote hummoral immunity, counterbalancing Th1 and, consequently, acting as anti-inflammatory molecules [15].

Periodontal disease is a complex condition. The dominance of a hummoral-type response is suggested in chronic lesions [12], but there is room to speculate that, in the early stages of the disease, cell-mediated reactions interfere with, or even direct, the disease progression. Analyses of cytokine profiles (i.e. type and levels of both Th1 and Th2) in periodontal tissues affected by chronic periodontitis have been performed using distinctly different strategies, such as *in situ* hybridization and immunohistochemistry [13], ELISA [7,9,11], RT-PCR [10,12] and more recently with cytometric bead arrays (CBA) [6,8]. Such studies in aggressive periodontitis are more scarce [9,10] and the CBA methodology has not been used so far in these patients. Some controversial results have been found, for example, elevated levels of IL-10 [6,9,13] and decreased levels of IL-2 [6,13] and IFN-γ [13] were observed in periodontitis compared to healthy controls. IL-4 was significantly present in aggressive [13] but not in chronic periodontitis [11]. IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ were not statistically significant in chronic periodontitis compared to a control group [8].

In order to further contribute to the characterization of the cytokine profile presented by periodontitis patients, especially those with the aggressive form of the disease, we evaluated the Th1/Th2 cytokine profile, defined by the detection of IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ in plasma from patients with aggressive and chronic periodontitis, using a CBA.

Materials and methods

Subjects

Fifty-six patients were selected from the Department of Periodontology at the Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil. Their age ranged from 13–64 years. After clinical examination, the patients who fulfilled the clinical inclusion criteria (see below) were invited to participate in the present study, which was submitted to and approved by the UFRGS ethics committee (Protocol Nr.: 47/05). All patients and individuals from the control group were thoroughly informed about the purpose and methods of the study and written consent was obtained from each of them.

Subjects were classified into the following groups: healthy control ($n = 18$), chronic ($n = 19$) and aggressive periodontitis ($n = 19$). Control healthy subjects had at least 20 teeth in the mouth, a gingival bleeding index less than 20%, attachment loss and/or probing depth in proximal sites ≤ 3 mm [16]. All periodontal examinations were performed by post-graduated periodontologists. Chronic periodontitis subjects were aged between 35–60 years and had at least 20 teeth, attachment loss ≥ 4 mm in at least 10 teeth, probing depth ≥ 6 mm in at least five teeth and periodontal bleeding in at least 10 teeth. Aggressive periodontitis subjects were those who had four or more teeth with attachment loss ≥ 4 mm in individuals between 14–19 years old or ≥ 5 mm in individuals between 20–29 years old [5]. A questionnaire was applied to all participants regarding their educational level, smoking habits and general health. For the present research, individuals diagnosed with human immunodeficiency virus and/or diabetes, pregnant or lactiferous women and anyone who took immunomodulatory/anti-inflammatory/antibiotics drugs in the 6 months prior to the study were excluded.

Sample collection

Blood samples (15 mL) were obtained by venipuncture from each patient using BD™ Vacutainer tubes (BD Diagnostics, Franklin Lakes, NJ, USA) which were immediately centrifuged in order to isolate the plasma. Plasma samples were aliquoted and stored at $-20°C$ until manipulation.

CBA analysis

The concentration of plasma cytokines was determined by means of flow cytometry using the BD™ CBA Human Th1/Th2 Cytokine Kit I (BD Biosciences, San Jose, CA, USA), a multiplex immunoassay which quantitatively measures soluble analytes on the basis of their different fluorescence intensities [6,8,17]. The CBA kit employed allows for the discrimination of the following cytokines: IL-2, IL-4, IL-5 and IL-10, as well as IFN-γ and TNF-α. The detection limits were as follows: IL-2, 2.6 pg/ml; IL-4, 2.6 pg/ml; IL-5, 2.5 pg/ml; IL-10, 2.8 pg/ml; IFN-γ, 7.1 pg/ml and TNF-α, 2.8 pg/ml. Sample processing and data analysis were performed according to the manufacturer's instructions. Sample data were acquired using a FACSCalibur flow cytometer (BD Biosciences).

Statistical analysis

The unit of analysis was established as the individual and α was set at 0.05. Patient data was compared with

Table I. Characteristics of the studied subjects.

	Periodontitis group			
	Healthy group	Chronic periodontitis	Aggressive periodontitis	*p*-value
Male/female gender, %	12/6 (66.7)	14/5 (73.7)	16/3 (84.2)	0.462
Age, mean (SD)	28.8 (4.36)[a]	49.4 (9.76)[b]	35.6 (8.48)[b]	0.031*
Ethnic group, %				0.204
Caucasian	18 (100)	16 (84.2)	16 (84.2)	
Non-Caucasian	0 (0.0)	3 (15.8)	3 (15.8)	
Smoking status, %				0.366
Non-smoker	14 (77.8)	11 (57.9)	11 (57.9)	
Smoker/former smoker	4 (22.2)	8 (42.1)	8 (42.1)	

*Statistically different at 0.05, different letters mean statistical difference.

ANOVA test for age and Chi-squared test for gender, ethnic group and smoking status. Concentration of cytokines in pg/ml was presented as median and percentiles 25 and 75 and compared using Kruskal-Wallis test. Spearman rho coefficient was used to correlate between cytokines in healthy, chronic and aggressive periodontitis groups and Pearson's correlation test to ascertain the relationship between age and each cytokine within the three studied groups. A value was considered zero when the level of the cytokine was below the methodological limit of detection and the sample was not excluded from the analysis. SPSS Statistics® 18.0 software was used to analyze the data.

Results

General characteristics of the subjects included in the study are presented in Table I. There was no statistical difference between the cytokines concentrations when the three groups were compared (Table II). However, some trends could be observed. IL-5 and IL-10 levels were lower in patients with aggressive (0.0 and 0.0, respectively) when compared to chronic periodontitis (1.2 and 1.9, respectively) and with healthy controls (0.6 and 0.0, respectively) ($p = 0.060$ and $p = 0.097$, respectively). Besides, IFN-γ levels decreased from healthy to chronic and to aggressive periodontitis groups, respectively (1.8, 1.4, 1.1) ($p = 0.435$). Chronic periodontitis patients presented higher levels of all cytokines when compared to healthy individuals, except IFN-γ (Table II). The pattern of cytokine profiles showed a high individual variability. Pearson's correlation analysis did not detect any relationship between age and cytokine levels when the three studied groups were analyzed (data unshown).

Tables III, IV, V describe the correlations between plasma cytokines of all the studied groups. Spearman rho coefficients between IL-2/IL-4 and IL-2/IL-10 were stronger in healthy individuals (0.938 and 0.669, respectively) compared with chronic (0.746 and 0.532, respectively) and aggressive periodontitis groups (0.395 and 0.266, respectively). A similar pattern was observed when IL-2/IFN-γ cytokines were analyzed, with the weakest correlation observed in the aggressive periodontitis group (0.600). When compared to the healthy (0.812) and chronic (0.845) groups, IL-4/IL-5 correlation was weaker (0.459) in the aggressive periodontitis group.

Table II. Concentration of plasma cytokines in pg/ml (median and percentiles 25 and 75) in chronic and aggressive periodontitis groups compared with healthy individuals.

	Periodontitis			
	Healthy group	Chronic periodontitis	Aggressive periodontitis	*p*-value
IL-2	0.6 (0.0–2.3)	1.6 (0.0–2.2)	1.4 (0.0–1.8)	0.592
IL-4	1.5 (0.0–2.6)	1.9 (0.0–2.5)	1.5 (0.0–2.0)	0.645
IL-5	0.6 (0.0–1.5)	1.2 (0.0–1.4)	0.0 (0.0–0.0)	0.060
IL-10	0.0 (0.0–2.4)	1.9 (0.0–3.0)	0.0 (0.0–1.5)	0.097
TNF-α	0.7 (0.0–2.9)	2.1 (0.0–3.1)	1.6 (0.0–2.4)	0.400
IFN-γ	1.8 (0.0–3.2)	1.4 (0.0–3.2)	1.1 (0.0–2.1)	0.435

p-value corresponds to an all-groups comparison by Kruskall Wallis test.

Table III. Spearman rho coefficients between cytokines in the healthy group.

	IL-2	IL-4	IL-5	IL-10	TNF-α	IFN-γ
IL-2						
IL-4	0.938					
IL-5	0.813	0.812				
IL-10	0.669	0.554	0.637			
TNF-α	0.597	0.526	0.703	0.703		
IFN-γ	0.733	0.801	0.772	0.772	0.737	

Table IV. Spearman rho coefficients between cytokines in the chronic periodontitis group.

	IL-2	IL-4	IL-5	IL-10	TNF-α	IFN-γ
IL-2						
IL-4	0.746					
IL-5	0.644	0.845				
IL-10	0.532	0.580	0.720			
TNF-α	0.741	0.762	0.738	0.792		
IFN-γ	0.655	0.751	0.744	0.807	0.846	

Table VI shows that the cytokine concentration in plasma samples was above the methodological detection limit in not all cases.

Discussion

In the present study, the expression of cytokines that define Th1 and Th2 immune response types (IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ) was compared in plasma of individuals with different forms of periodontitis. No statistical difference could be observed between groups. We used in aggressive periodontitis patients the CBA technique, a flow cytometry multiplex assay that has clear advantages compared to conventional ELISA, such as obtaining a standard curve for each analyte studied from a single set of diluted standards. Besides, it allows measuring simultaneously six cytokines belonging to the Th1 and Th2 profile, minimizing methodological errors. When the cytokines levels were below the methodological limit we considered as zero and included the sample in the analysis [6]. In this case, zero doesn't mean an absolute number, but rather stands for a value inferior to the detection limit of the test.

Our results are in accordance with a previous work that investigated the systemic levels of some inflammatory mediators, such as IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ, with CBA in patients with chronic periodontitis and healthy controls [8]. Using the same methodology, the authors have not observed statistical difference when all the cytokines above were

Table V. Spearman rho coefficients between cytokines in the aggressive periodontitis group.

	IL-2	IL-4	IL-5	IL-10	TNF-α	IFN-γ
IL-2						
IL-4	0.395					
IL-5	0.681	0.459				
IL-10	0.266	0.557	0.674			
TNF-α	0.424	0.327	0.521	0.619		
IFN-γ	0.600	0.668	0.521	0.543	0.591	

Table VI. Percentage of detection when the level of each cytokine was above the detection limit in healthy, chronic and aggressive periodontitis groups.

	IL-2	IL-4	IL-5	IL-10	TNF-α	IFN-γ
Healthy	50	56	56	39	56	72
Chronic periodontitis	63	68	21	26	47	53
Aggressive periodontitis	63	63	58	53	68	53

measured. However, this study has not included an aggressive periodontitis group.

Recently, a new Th cell lineage capable of producing IL-17, the Th17 cells, has been described. This cytokine is an important regulator of neutrophil and macrophage migration and subsequent pathogen elimination. It acts over osteoblast enhancing the receptor activator nuclear factor kappa B ligand (RANKL) expression, inducing directly the differentiation of osteoclast progenitors in mature osteoclast and is able to stimulate C-reactive protein production [18,19]. The Th17 profile has not been included in our analysis since the present manuscript corresponds to an experimental approach designed before the availability of a commercial kit including testing for IL-17 levels. Nevertheless, to our knowledge, gingival crevicular fluid levels of IL-17 were recently assessed comparing aggressive periodontal patients and healthy controls and no differences were observed in the total amount of this cytokine between the groups [20]. Considering that Ay et al. [20] performed their analyses on gingival crevicular fluid, a compartment close to the local of inflammation and showing an absence of correlation among disease and IL-17 total amount, we believe that similar results would be achieved when assessing plasma cytokine levels. However, we cannot rule out the possibility of different plasma IL-17 levels among periodontitis patients and controls and a new set of patients is intended to be collected in order to approach that [18,20].

In the present study, a trend to lower levels of IL-5 and IL-10 in aggressive periodontitis (0.0 and 0.0, respectively) was observed when compared to both chronic periodontitis patients (1.2 and 1.9, respectively) and healthy controls (0.6 and 0.0, respectively) ($p = 0.060$ and $p = 0.097$, respectively). Low levels of IL-5 and IL-10 (typical Th2-type response cytokines) observed in the aggressive periodontitis group could lead to a defective control of the immune responses directed against pathogens, consequently resulting in exacerbated inflammation. Through cytokine mRNA analysis, Suárez et al. [10] observed the expression of IL-5 and IL-10 in biopsies from healthy and periodontitis patients, although this expression was absent from the diseased tissue obtained from patients with aggressive periodontitis, which comes in favor of our data. Górska et al. [11] analyzed cytokine concentrations in inflamed gingival

tissues and serum samples from patients with severe chronic periodontitis and compared them to healthy controls. Serum samples showed high individual variability of cytokine profiles, and no association between cytokine concentrations and clinical parameters of periodontitis was found. Nevertheless, the authors point out that, although the IL-10 levels were generally low or even undetectable in serum, the frequency of individuals expressing IL-10 positive cells was much higher in healthy gingival tissues as compared to chronic periodontitis patients, again suggesting that local expression of this molecule can control inflammation. Also, in accordance with our data, Garlet et al. [12] observed, through RT-PCR, lower IL-10 expression in gingival biopsies from patients presenting with aggressive periodontitis.

In addition, we found lower levels of IFN-γ in the periodontitis group compared to healthy controls, in agreement with the findings of Lappin et al. [13]. On the other hand, we observed a higher level of TNF-α in periodontitis patients compared to healthy controls Andrukhov et al. [6].

IL-2/IL-4 Spearman rho coefficient was stronger in healthy controls compared to the periodontitis group. IL-2 plays an essential role in Th2 priming and stabilizes the accessibility of the *Il4* gene, and STAT5, a key transducer of IL-2 function [21]. We found a weak correlation between IL-4/IL-5 (0.459) in the aggressive periodontitis group. The chemoattraction potential of the cytokines produced by Th1 could favor bone resorption and disease progression. As pointed out by Garlet et al. [12], the chemoattracting characteristics of IL-4 and IL-10 producing cells could control the potentially destructive Th1 response. As IL-10 is associated with suppression of bone resorption [22,23], its low expression in patients with aggressive periodontitis could account for the more severe form of disease.

The present study shows a high variability of cytokines levels in plasma. Recently, it has been suggested that, in periodontitis, this variability and the lower frequency of its detection may contribute to determining the presence and/or severity of the disease [8]. Although Górska et al. [11] affirm that cytokine serum levels are not good disease indicators in periodontitis and that such results may lead to controversial conclusions, the occurrence of an increase in T-cell numbers in peripheral blood and the homing of these cells to the gingiva are well established during the disease process [13]. Furthermore, the level of cytokines in plasma can represent a possible spill-over to the circulation of cytokines produced locally in the inflamed tissues [9]. There is evidence that periodontitis can be associated through this mechanism to an increased risk of a series of systemic conditions such as cardiovascular disease [24] and pregnancy adverse events [25].

Further studies with an increased number of subjects may show more precise results for the relationship between cytokines and periodontitis. However, aggressive periodontitis is a rare condition and few specific publications are found in the literature. In a representative sample of young individuals (14–29 years old) from the metropolitan Porto Alegre/Brazil region, 5.5% of the subjects presented aggressive periodontitis [5].

Our results indicate no difference between systemic levels of Th1 and Th2 cytokines between all groups. Since Th2 cytokines are involved in the regulation of inflammatory responses, the observed trend towards low levels of Th2 cytokines in aggressive periodontitis patients could suggest a contribution to the development of such an exacerbated manifestation of this disease.

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5 CONSIDERAÇÕES FINAIS

A influência de fatores imunogenéticos na periodontite já foi avaliada por vários grupos de pesquisa. Entretanto, os resultados são inconclusivos por inúmeras razões, das quais podemos destacar os critérios de inclusão dos pacientes em relação as suas condições clínicas, o número amostral, a etnia e a metodologia.

Os polimorfismos de genes envolvidos nas respostas imunológicas e inflamatórias, como o HLA-G, vêm sendo estudados na tentativa de elucidar características genéticas individuais que possam caracterizar um genótipo de suscetibilidade para determinados pacientes. Nosso estudo foi o pioneiro em avaliar dois polimorfismos correlacionados com o gene HLA-G (ins /del de 14 pb e C/G +3142) em pacientes com periodontite. Sabemos das limitações relacionadas ao tamanho amostral, uma vez que, para resultados mais representativos da população, deveríamos ampliar o número de indivíduos participantes. Acreditamos que, para um estudo preliminar, obtivemos resultados que nos permitem afirmar que o alelo de deleção em indivíduos homozigotos representa um papel de suscetibilidade na patogênese de pacientes com PC. Entretanto, não foram observadas diferenças significativas na análise do polimorfismo de 14 pb considerando o grupo com PA comparado ao controle, assim como para o polimorfismo +3142 C/G, independente dos grupos estudados.

Em relação ao perfil de citocinas Th1/Th2, existem trabalhos com metodologia semelhante a utilizada em nosso estudo, todavia, não realizados em pacientes com PA. Apesar de não observarmos diferenças significativas na expressão de ambos os perfis de citocinas (IL-2, IL-4, IL-5, IL-10, TNF-α e IFN-γ), verificamos uma tendência a menores níveis de IL-5 e IL-10, o que poderia contribuir para um quadro exacerbado da doença em pacientes com PA.

Conforme mencionado na introdução desta tese, o HLA-G poderia ter implicações importantes no desenvolvimento de situações mediadas pelas respostas Th1/Th2. Trabalhos futuros deverão ser realizados aferindo, simultaneamente, a expressão de HLA-

G e a IL-10, confirmando os resultados sugeridos na literatura em relação a outras condições inflamatórias. Acreditamos que a polarização Th2 induzida pelo HLA-G proporcionaria um aumento desta molécula proporcionalmente às citocinas com perfil anti-inflamatório.

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ANEXO 1

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA EM PESQUISA

Ofício 272/08-CEP

Porto Alegre, 25 de março de 2008.

Senhor(a) Pesquisador(a):

O Comitê de Ética em Pesquisa da PUCRS apreciou e aprovou seu protocolo de pesquisa registro CEP 08/04085, intitulado: "**Avaliação genética de pacientes com periodontite agressiva através da análise de polimorfismo de genes associados a processos inflamatórios".**

Sua investigação está autorizada a partir da presente data.

Relatórios parciais e final da pesquisa devem ser entregues a este CEP.

Prof. Dr. José Roberto Goldim
COORDENADOR DO CEP-PUCRS

ANEXO 2

UFRGS
Universidade Federal do Rio Grande do Sul — Faculdade de Odontologia

COMITÊ DE ÉTICA EM PESQUISA

RESOLUÇÃO

O Comitê de Ética em Pesquisa e a Comissão de Pesquisas da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul analisaram o Projeto:

Número: 47/05

Título: AVALIAÇÃO GENÉTICA DE PACIENTES COM PERIODONTITE AGRESSIVA ATRAVÉS DA ANÁLISE DE POLIMORFISMOS DE GENES ASSOCIADOS A PROCESSOS INFLAMATÓRIOS

Investigador(es) principal(ais): Professores Rui V. Oppermann, José Artur BogoChies e C.D. Leticia Algarves Miranda

O Projeto foi aprovado na reunião do dia 06/12/05, Ata nº 11/05 do Comitê de Ética em Pesquisa e da Comissão de Pesquisas, da UFRGS, por estar adequado ética e metodologicamente e de acordo com a Resolução 196/96 do Conselho Nacional de Saúde.

Porto Alegre, 09 de dezembro de 2005.

Prof. Marisa Maltz
Coordenadora do Comitê de Ética em Pesquisas

Profa. Heloísa Emília Dias da Silveira
Coordenadora da Comissão de Pesquisas