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IMUNODETECÇÃO DO VEGF E DAS ANGIOPOIETINAS 1 E 2 EM LÍQUEN PLANO ORAL

MÁRCIA RODRIGUES PAYERAS

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IMMUNODETECTION OF VEGF AND ANGIOPOIETINS 1 AND 2 IN ORAL LICHEN PLANUS

Tese apresentada como requisito para obtenção do Título de Doutor pelo Programa de Pós-Graduação em Odontologia da Pontifícia Universidade Católica do Rio Grande do Sul. Área de Concentração em Estomatologia Clínica.

Orientadora: Profa. Dra. Fernanda Gonçalves Salum

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RESUMO

Neste estudo foi investigada a imunodetecção do VEGF e das angiopoietinas (ANG) 1 e 2, bem como o número de vasos sanguíneos em espécimes de líquen plano oral reticular (LPOR) e atrófico-erosivo (LPOAE). Além disso, foram incluídos os grupos-controle hiperplasia fibroepitelial (HFO), displasia epitelial (DEO) e carcinoma espinocelular (CEO) orais, a fim de encontrar uma relação entre esses fatores de crescimento angiogênicos e comportamento biológico. Foram obtidos espécimes de biópsias do arquivo do Serviço de Estomatologia e Prevenção do Câncer Bucomaxilofacial do Hospital São Lucas da PUCRS, os quais foram distribuídos nos grupos LPOR (n=21); LPOAE (n=11); HFO (n=10); DEO (n=10); CEO (n=10) e, posteriormente, submetidos ao processamento imunoistoquímico com os anticorpos primários anti-VEGF, anti-ANG-1 e anti-ANG-2. Os resultados não revelaram diferença significativa na imunodetecção da ANG-1 e ANG-2 entre os grupos líquen plano oral (LPO). No entanto, o grupo LPOR apresentou imunodetecção significativamente superior do VEGF (p=0,01) comparado ao grupo LPOAE. Com relação ao número de vasos sanguíneos, a comparação entre os grupos LPO não mostrou diferença significativa entre ambos (p=0,393). Na análise comparativa com os grupos-controle, os casos de LPOR e LPOAE foram considerados em conjunto, formando o grupo LPO (n=32). A comparação da imunodetecção dos fatores de crescimento entre o grupo LPO com cada grupo-controle revelou valores significativamente superiores da ANG-1 no grupo LPO em relação ao grupo HFO (p=0,043) e ausência de diferença significativa nos valores da ANG-2. A imunodetecção do VEGF foi significativamente superior no grupo LPO em relação ao grupo HFO (p=0,003) e inferior quando comparada ao grupo CEO

(p=0,032). Não foi observada correlação entre os fatores de crescimento avaliados nos diferentes grupos estudados. Os resultados observados na imunodetecção do VEGF, ANG-1 e ANG-2 e a falta de diferença significativa no número de vasos entre os grupos LPOR e LPOAE indicam que a angiogênese no LPO possa não estar associada às diferentes formas clínicas desta doença. A falta de correlação entre estes fatores de crescimento nos diferentes tipos de lesões estudadas indica o envolvimento de outras citocinas pró-angiogênicas, além daquelas avaliadas neste estudo, na neoformação vascular e reflete a complexidade deste processo. Por outro lado, a imunodetecção dos fatores de crescimento avaliados foi semelhante entre os grupos LPO e DEO, sugerindo que a angiogênese no LPO possa comportar-se de modo semelhante ao de lesões displásicas epiteliais.

Palavras-chave: Líquen plano oral. Angiogênese. Fator de crescimento endotelial vascular. Angiopoietina-1. Angiopoietina-2.

ABSTRACT

In this study we investigated the immunodetection of VEGF, ANG-1 and ANG-2, as well as the number of blood vessels in specimens of reticular (ROLP) and atrophic-erosive oral lichen planus (AEOLP). In addition, were included the control groups oral epithelial hyperplasia (OFH), oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC) in order to find a relation between those angiogenics growth factors and biological behavior. Biopsy specimens diagnosed at the Oral Medicine Division of PUCRS, Brazil, were selected and distributed into the following groups ROLP (n=21); AEOLP (n=11); OFH (n=10); OED (n=10); and OSCC (n=10). They were subjected to immunohistochemical processing with primary antibodies anti-VEGF, anti-ANG-1 and anti-ANG-2. The results do not revelead significant difference in the immunodetection of ANG-1 and ANG-2 between the OLP groups. The ROLP group showed significantly higher immunodetection of VEGF (P=0.01) compared with the AEOLP group. There was no significant difference in the number of blood vessels between the OLP groups (P=0.393). In comparative analysis of OLP with control groups, ROLP and AEOLP cases were considered together, forming the OLP group (n=32). The comparison of immunodetection of those growth factors between the OLP group and each control group showed significantly higher values of ANG-1 in the OLP group compared to the OFH group (P=0.043) and no significant difference in ANG-2. VEGF immunodetection in the OLP group was significantly higher than in the OFH group (P=0.003) and significantly lower compared to the OSCC group (P=0.032). No correlation was observed between growth factors evaluated in the different groups studied. The results of immunodetection of VEGF, ANG-1 and ANG-2 and the lack of significant difference

in the number of blood vessels between ROLP and AEOLP groups indicate that angiogenesis in OLP can not be associated to the different clinical forms of this disease. The lack of correlation between the growth factors in the different lesions indicates the involvement of other pro-angiogenic agents in vascular neoformation and reflects the complexity of this process. Furthermore, immunodetection of growth factors evaluated was similar between OLP and OED, suggesting that angiogenesis in OLP may behave similarly to the epithelial dysplastic lesions.

Keywords: Oral lichen planus. Angiogenesis. Vascular endothelial growth factor. Angiopioetin-1. Angiopoietin-2.

LISTA DE TABELAS

ORAL LICHEN PLANUS: FOCUS ON ETIOPATHOGENESIS

IMMUNODETECTION OF VEGF AND ANGIOPOIETINS 1 AND 2 IN ORAL LICHEN PLANUS

LISTA DE FIGURAS

ORAL LICHEN PLANUS: FOCUS ON ETIOPATHOGENESIS

IMMUNODETECTION OF VEGF AND ANGIOPOIETINS 1 AND 2 IN ORAL LICHEN PLANUS

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

%	Por cento; Percent
°C	Degree Celsius
AEOLP	Atrophic-Erosive Oral Lichen Planus
ANG	Angiopoietina; Angiopoietin
AP	Activator Protein
CC	Subfamily of Chemokines
CCR	Subgroup of Chemokines Receptors
CD34	Cluster of Differentiation 34
CD4+ T	Helper T- Lymphocyte
CD8+ T	Suppressor T – Lymphocyte
CEO	Carcinoma Espinocelular oral
COX	Cyclooxygenase
DAB	Diaminobenzidine
DEO	Displasia Epitelial Oral
DNA	Deoxyribonucleic Acid
EGF	Fator de Crescimento Epidérmico; Epidermal Growth Factor
Fas L	Type II – Transmembrane protein
FGF	Fator de Crescimento de Fibroblasto; Fibroblast Growth Factor
Flt-1	Fms-related tyrosine kinase
GGT	Gamma Glutamyl Transpherase
HCV	Vírus da Hepatite C; Hepatitis-C Virus
HE	Hematoxylin and Eosin
HFO	Hiperplasia Fibroepitelial Oral
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigens
HPV	Vírus do Papiloma Humano; Human Papillomavirus
HSP	Heat Shock Protein
ICAM	Molécula de Adesão Intercelular; Intercellular Adhesion Molecule
IFN	Interferon
Ig	Immunoglobulin
IGF	Fator de Crescimento Semelhante à Insulina; Insulin-like Growth Factor

IHC	Immunohistochemistry
IL	Interleucina; Interleukin
ISH	In situ Hybridization
KDR	Kinase insert Domain Receptor
LCs	Langerhans Cells
LP	Líquen Plano; Lichen Planus
LPO	Líquen Plano Oral
LPOAE	Líquen Plano Oral Atrófico-Erosivo
LPOR	Líquen Plano Oral Reticular
MCs	Mastocyte Cell
MDA	Malondialdehyde
MVD	Densidade Microvascular; Microvessel Density
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinases
NFKB	Nuclear Factor KB
OED	Oral Epithelial Dysplasia
OFH	Oral Fibroepithelial Hyperplasia
OLP	Oral Lichen Planus
OLR	Oral Lichenoid Reaction
OMS	Organização Mundial da Saúde
OSCC	Oral Squamous Cell Carcinoma
PCR	Polymerase Chain Reaction
PDCs	Plasmacytoid Dendritic Cells
PDGF	Fator de Crescimento Derivado de Plaquetas; Platelet-Derived Growth Factor
pH	Potential Hydrogen
PI	Phosphatidlinositol
PIGF	Fator de Crescimento Placentário; Placental Growth Factor
RANTES	Regulated on Activation Normal T-cell Expressed and Secreted
RCA	Request Cytotoxic Activity
ROLP	Reticular Oral Lichen Planus
RT-PCR	Real Time Polymerase Chain Reaction
SD	Standart Deviation
SPSS	Statistical Package for Social Sciences
STAT	Signal Transducer and Activator of Transcription

TAA	Total Antioxidant Defense
TGF-α	Fator de Transformação do Crescimento - alfa; Transforming Growth Factor – alpha
TGF-β	Fator de Transformação do Crescimento – beta; Transforming Growth Factor – beta
Th	T Helper cell
Tie2	Receptor Tirosina Kinase Tie 2; Tyrosine Kinase Receptor Tie 2
TIMPs	Tissue Inhibitor of Metalloproteinase
TNF-α	Fator de Necrose Tumoral Alfa; Tumor Necrosis Factor-alfa
VCAM-1	Molécula de Adesão de Células Vasculares-1; Vascular Cell Adhesion Molecule-1
VEGF	Fator de Crescimento Endotelial Vascular; Vascular Endothelial Growth Factor
VEGFR	Receptor de Fator de Crescimento Endotelial Vascular; Vascular Endothelial Growth Factor Receptor
WHO	World Health Organization
WPBs	Weibel-Palade bodies
µm	Micrometer

SUMÁRIO

1 INTRODUÇÃO

O líquen plano (LP) é uma doença inflamatória crônica que apresenta manifestações em pele, couro cabeludo, unhas e mucosas.[1-3] A mucosa bucal é um dos sítios mais frequentemente acometidos e, muitas vezes, a única forma de manifestação da doença.[4] O líquen plano oral (LPO) acomete aproximadamente 1% da população,[5] apresentando predileção por pacientes do gênero feminino e de meia-idade.[1,6,7] Sua presença é incomum em crianças[2] e, aparentemente, não apresenta preferência por raça.[8]

Atualmente, pesquisas têm investigado a etiologia[9-12] e patogênese do LPO[6,7,13,14], bem como identificado alterações em proteínas que regulam a proliferação celular,[15-17] apoptose[18-20] e angiogênese,[21-24] visando entender os mecanismos relacionados com o comportamento biológico desta doença.

A angiogênese, processo pelo qual há formação de novos vasos a partir de estruturas vasculares pré-existentes, tem importante papel em condições fisiológicas e em diversas condições patológicas, como no desenvolvimento de doenças inflamatórias crônicas, crescimento de neoplasias e metástases, má formações vasculares e doenças cardiovasculares.[22,25,26] Esse processo desenvolve-se em múltiplas etapas tais como desestabilização, proliferação, migração, remodelamento e estabilização endotelial[26,27] e é controlado por numerosos fatores de crescimento e citocinas pró-angiogênicas, assim como por diversos inibidores endógenos de neovascularização.[28] Em situações fisiológicas, esses ativadores e inibidores encontram-se em equilíbrio, entretanto, em situações patológicas pode haver persistente ou excessiva ativação de agentes pró-angiogênicos.[26]

Um dos mais conhecidos reguladores da angiogênese é o Fator de Crescimento Endotelial Vascular (VEGF), cuja habilidade está em induzir a permeabilidade vascular,[29] bem como a proliferação e migração endotelial, etapas fundamentais para o desenvolvimento de novos vasos sanguíneos.[30-32] A capacidade deste fator de crescimento em aumentar a permeabilidade vascular contribui para que o mesmo desempenhe importante papel em processos inflamatórios, infiltração tumoral e metástases.[28]

A secreção do VEGF é determinada por vários tipos celulares como queratinócitos, macrófagos e outras células do sistema imune[31] e sua expressão é regulada por numerosos fatores de crescimento (EGF, TGF-α, TGF-β, IGF-1, FGF, PDGF), citocinas (TNF-α, lL-1α, lL-6, IL-8) e, principalmente, pela hipóxia.[28] O VEGF é uma família composta pelos fatores de crescimento derivados de plaquetas: VEGFA, VEGFB, VEGFC, VEGFD, VEGFE e PIGF.[28] O significado biológico destas diferentes formas ainda não está totalmente compreendido e dentre elas, o VEGFA, também conhecido na literatura como VEGF, é o mais comum[33] e considerado o principal regulador da angiogênese fisiológica e patológica.[30] O VEGFA consiste de pelo menos quatro isoformas: $VEGF_{121}$, $VEFG_{165}$, $VEGF_{189}$ e $VEGF_{206}$,[34] assim determinadas em função do número de aminoácidos que contém.[33] Dependendo de sua habilidade de ligarem-se à heparina, estas isoformas ficam acumuladas na matriz extracelular ou são liberadas, tornando-se acessíveis à interação com outros tipos celulares.[28] O $VEGF_{165}$, subtipo mais abundante *in vivo,* além de apresentar atividade metabólica mais potente, apresenta afinidade moderada pela heparina, estando presente na superfície celular e na matriz extracelular.[26,33,34]

As diferentes formas do VEGF ligam-se aos receptores tirosina quinase envolvidos no início da cascata de transdução do sinal: VEGFR-1, VEGFR-2 e

VEGFR-3.[25] Estes receptores foram inicialmente detectados em células do endotélio vascular, porém mais recentemente têm sido observados em células não vasculares tais como as células epiteliais.[34] O VEGFA liga-se aos receptores VEGFR-1 (Flt-1), onde provoca fraca fosforilação e VEGFR-2 (KDR), por meio do qual induz os seus principais efeitos biológicos.[28,30]

Outros fatores de crescimento essenciais para a formação vascular são as Angiopoietinas (ANG), família composta por quatro subtipos denominados ANG-1, ANG-2, ANG-3 e ANG-4. As ANG-1 e ANG-2 são as mais conhecidas e melhor caracterizadas,[35] apresentando papel-chave na quiescência e regulação da integridade vascular.[36] A ANG-1 é expressa em células mesenquimais, principalmente perícitos, células musculares lisas, fibroblastos e em algumas células tumorais, agindo de maneira parácrina sobre o endotélio.[37] A ANG-2 é expressa em células musculares lisas, epitelais[38] e em células endoteliais, onde está estocada nos *Weibel-Palade bodies* (WPBs),[36] atuando por um mecanismo autócrino.[35]

A produção da ANG-1 e ANG-2 é induzida pela hipóxia e por várias citocinas, incluindo o VEGFA, PDGF-B e TNF-α.[39-42] Ambos os fatores de crescimento ANG-1 e ANG-2 ligam-se aos receptores tirosina quinase Tie2, expressos no endotélio vascular, porém somente a ANG-1 induz a fosforilação deste receptor e sua ativação, com consequente transdução de sinal.[35] A ligação da ANG-2 com o receptor Tie2 inibe o efeito da ANG-1 e dessa forma, a ANG-2 é considerada antagonista da ANG-1,[35,43] provocando regressão de vasos neoformados e apoptose de células endoteliais.[37] No entanto, alguns estudos identificaram a ANG-2 como agonista para o receptor Tie2, estimulando a angiogênese,[44] quando este receptor estiver expresso em células não-endoteliais[43] ou quando o VEGF também estiver presente.[45]

Ao contrário do VEGF, as angiopoietinas são mediadores angiogênicos cuja ação não induz atividade mitótica em células endoteliais.[46] Apesar de sua atividade biológica não ser totalmente conhecida, sabe-se que a ANG-1 é constantemente secretada em situações de quiescência vascular, bem como durante o processo de maturação vascular, etapa mais tardia da angiogênese, uma vez que promove interação entre as células endoteliais e a matriz extracelular circundante.[25,36,46] Além disso, a ANG-1 apresenta efeito protetor sobre o endotélio por limitar a sua ativação por citocinas exógenas, o que determina que este mediador exiba propriedades anti-inflamatórias e desempenhe importante função como regulador da inflamação.[36] Por outro lado, a ANG-2 desestabiliza o endotélio vascular, tornando-o suscetível a diversos estímulos exógenos produzidos por outras citocinas como o VEGF, que induzem a angiogênese e a resposta inflamatória.[36] Ressalta-se que o efeito produzido por esses mediadores sobre a vasculatura depende do balanço existente entre ANG-1 e ANG-2, bem como da presença de outros agentes exógenos como o VEGF e o TNF.[36]

Evidências atuais têm demonstrado que a angiogênese é um evento precoce da transformação maligna. Johnstone e Logan[47] constataram aumento significativo da imunodetecção do VEGF em displasia epitelial e carcinoma espinocelular em relação à mucosa bucal normal, sugerindo que este mediador tem importante papel na manutenção do suprimento sanguíneo durante a transformação maligna. Em doenças inflamatórias crônicas, a angiogênese apresenta papel-chave na patogenia, não somente por originar novos vasos, que aumentarão a oxigenação e a atividade metabólica tecidual, mas por elevar notavelmente o volume de células envolvidas no processo inflamatório.[48] Atualmente, estudos têm demonstrado que mediadores angiogênicos como as ANG-1 e ANG-2 atuam no desenvolvimento e perpetuação de

doenças inflamatórias crônicas como psoríase, artrite reumatóide e as intestinais,[46,49-51] porém a influência desses mediadores no LPO ainda não foi investigada.

No LPO, acredita-se que a rica vascularização ocorra provavelmente em função da hipóxia presente no estroma, originada pela proliferação de linfócitos,[22] e que a angiogênese possa desempenhar um papel distinto nas diferentes formas clínicas dessa doença.[21] Tao et al.[21] observaram que o LPO erosivo apresentou maior densidade microvascular (MVD) em comparação às lesões reticulares e à mucosa bucal normal. No entanto, não foi observada diferença significativa na expressão do VEGF entre as formas reticulares e atrófico-erosivas do LPO. Por outro lado, esta diferença foi encontrada em um estudo mais recente, que demonstrou níveis séricos de VEGF superiores em pacientes com LPO erosivo.[23] Mittal et al.[24] encontraram diferenças significativas na MVD entre lesões de LPO e mucosa bucal normal, bem como entre lesões de LPO reticular e erosivo. Dessa forma, a angiogênese foi mais pronunciada nas lesões erosivas, sugerindo que possa agir não somente na etiopatogenia, mas também na progressão desta doença.

Scardina et al.[22] investigaram a imunodetecção do CD34, do VEGF e das moléculas de adesão VCAM-1 (Molécula de Adesão de Células Vasculares) e ICAM-1 (Molécula de Adesão Intercelular) em pacientes com LPO. Os autores encontraram valores significativamente superiores na imunodetecção de todos os anticorpos em pacientes com LPO quando comparados aos controles saudáveis, mostrando que uma significativa neoformação vascular ocorre nesta doença.

O papel da angiogênese no LPO, bem como nos seus diferentes padrões clínicos permanece incerto. Portanto, estudos são necessários para investigar o mecanismo de neoformação vascular nesta doença.

2 PROPOSIÇÃO

- Avaliar a imunodetecção do VEGF, ANG-1 e ANG-2 em espécimes de líquen plano oral.
- Comparar a imunodetecção do VEGF, ANG-1 e ANG-2 entre espécimes de líquen plano oral reticular e atrófico-erosivo.
- Comparar o número de vasos sanguíneos entre espécimes de líquen plano oral reticular e atrófico-erosivo.
- Comparar a imunodetecção do VEGF, ANG-1 e ANG-2 entre espécimes de líquen plano oral e hiperplasia fibroepitelial, displasia epitelial e carcinoma espinocelular orais.
- Correlacionar a imunodetecção do VEGF, ANG-1 e ANG-2 em espécimes de líquen plano oral, hiperplasia fibroepitelial, displasia epitelial e carcinoma espinocelular orais.

3 ARTIGO DE REVISÃO

ORAL LICHEN PLANUS: FOCUS ON ETIOPATHOGENSIS

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ORAL LICHEN PLANUS: FOCUS ON ETIOPATHOGENESIS

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Abstract

Lichen planus is a chronic mucocutaneous inflammatory disease, which frequently affects the oral mucosa of white females over 40 years old. Its etiology remains uncertain and the pathogenesis is still the object of much speculation. The present paper presents the most well known antigens, and describes the action of different cells and proteins associated with the development of that disease, as well as the possible agents involved with its malignant transformation. Different external agents, especially virus, and internal agents, like stress, and the *heat shock protein* antigen expression, associated or not, can alter the basal keratinocytes of the oral mucosa making them susceptible to apoptosis by CD8+ cytotoxic T cell as well as activate matrix metalloproteinase and mast cell degranulation, which produce a great range of inflammatory mediators and cytokines determining the clinical onset of the disease. Regarding carcinogenesis, since it is a complex process and presents multifactorial origin, it is believed that there may be a synergism between intrinsic, such as inflammation mediators, and extrinsic agents (tobacco, alcohol, viral infections) for the OLP malignant transformation to occur. However, further studies are needed to better understand the origin, pathogenesis and process of malignant transformation of OLP.

Introduction

Lichen Planus (LP) is a chronic mucocutaneous inflammatory disease that frequently affects the oral mucosa.[1,2] It tends toward white and middle-aged female patients.[3-5] Clinically, oral lichen planus (OLP) can present six different patterns: papule, reticular, plaque, atrophic, erosive and bullous[6] (Figure 1), each showing

specific characteristics and appearing in either isolated or associated forms.[1] Among those, the reticular is the most prevalent type characterized by the presence of Wickham striae, which are typically symmetric, bilateral, asymptomatic, and predominantly found in the buccal mucosa. The erosive form, although less frequent, presents greater clinical significance as the lesions are usually symptomatic, ranging from a slight discomfort to episodes of intense pain.[7] In the latter, the use of topical steroids are the first line to reduce symptoms and to improve the patients' quality of life. However, in case of persistent lesions systemic steroids are indicated.[8] Moreover, in situations of poor response of to the treatments above, alternative treatments ones can still be used,[8] such as calcineurin inhibitors,[9] antioxidants,[10] biologic therapies,[11] Photo Dynamic Therapy[12] and Laser Therapy.[13] OLP manifestations can persist for years alternating between periods of quiescence and exacerbation.[1]

Upon histopathological examination, OLP lesions present hyperkeratosis (in papular, plaque and reticular forms), hydropic degeneration of the basal cell layer of the epithelium and dense well-defined lymphocyte infiltrate, predominantly T, in the superficial subjacent connective tissue. The width of the spinous layer varies and the interpapillary ridges might not be present or be hyperplasic, but are often cuspidal or saw-tooth.[7,14-16]

The follow up of OLP patients has shown some evidence of the disease malignant potential.[4,5,14,17-19] Despite some controversies on this issue, the OLP is actually a potentially malignant disorder.[20] Thus, a long-term monitoring of those patients is recommended, although the consultation frequency has not been established yet.[2,21]

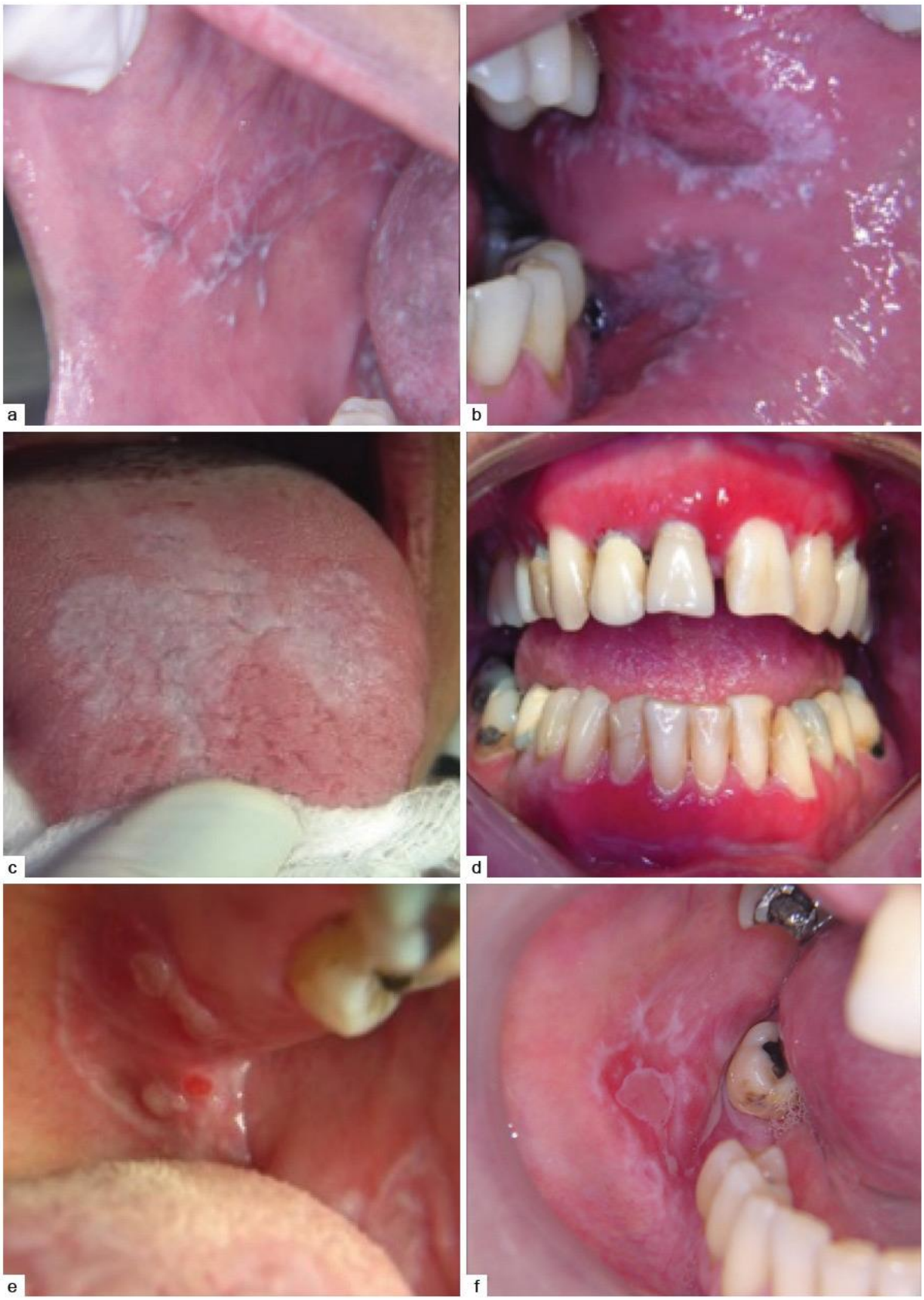

Figure 1: (a): Reticular OLP; (b): Papule OLP; (c): Plaque OLP; (d): Atrophic OLP; (e): Bullous OLP; (f): Erosive OLP.

OLP etiology and pathogenesis has not been totally understood, however, some external and/or internal antigens have been suggested to trigger this disease, and different development mechanisms have also been hypothesized. Recently, Roospharee et al. (2010)[22] conducted a review discussing the various hypotheses on OLP pathogenesis. In the present review, besides the pathogenesis discussed through the performance of different cell types, chemokines and metalloproteinases, the etiology of that disease and possible agents involved in the malignant transformation of OLP will be approached.

Etiology

The etiology of the OLP remains uncertain, but some evidence points out that the disease is an immunological process triggered by an antigen that alters the basal keratinocytes of the oral mucosa making them susceptible to cell immune response. It induces the activation of $CD4^+$ T and $CD8^+$ T lymphocytes and cytokines production**,** such as interleukin-2 (IL-2), interferon gama (IFN-γ) and tumor necrosis factor (TNF), which determine the keratinocytes apoptosis.[1,2,23-26]

The antigen that triggers this inflammatory response is still unknown, it might have an intrinsical or an extrinsical origin,[2] besides, there are controversies whether one or two are involved.[15] In some cases, extrinsical antigens that include dental restoration[27-29] and drugs, specially antimalarials,[30] cardiovascular agents,[31-33] gold salts,[34] nonsteroidal anti-inflammatory,[31,35] hypoglicemics[33] have been identified. In such cases, in spite of presenting lesions with clinical and microscopic features very similar to the OLP ones,[1,36,37] they are preferentially called oral lichenoid reaction (OLR).[21] Therefore, the diagnosis must be based on the recognition of clinical

alterations as well as on carrying out an interview with the objective of observing a possible cause-effect relationship.[38,39]

Several reports have pointed out a possible association between OLP and viral infections. Four human herpes virus families subtypes have been associated with oral manifestations of lichen planus: Herpes simplex,[40] Epstein-Barr,[40,41] Cytomegalovirus[42,43] and Herpes virus 6.[43,44] However, some doubts remain whether these agents are associated with the OLP or whether the infection superimposes the lesions already in existence.[25]

The most extensively investigated viruses in the OLP etiology are the human papillomavirus (HPV) and the hepatitis-C virus (HCV). Present investigations have shown that, most of the time, a high identification of HPV in OLP lesion (Table 1) and recent systematic review shows the strong association between HPV and OLP.[54] Furthermore, it has been proved that the HPV prevalence increased gradually with increasing severity of the lesions.[46,51,55] These findings suggest that HPV may play some etiological role in OLP, but also be associated with the malignant progression of potentially malignant oral disorders. However, it is believed that frequent ulcerative lesions in OLP that make it more susceptible to HPV infection,[54] or the chronic use of steroids could enhance replication of HPV virus.[54,55] Therefore, it is impossible to believe that the isolated presence of this virus is associated with the OLP etiology, as well as, with its malignant transformation, but that its presence could enhance the effect of carcinogens agents, increasing the malignant transformation risk.[51,56]

Table 1: The prevalence of HPV in OLP cases published in the past 10 years (Pubmed).

Reference	N	Detection Methods	Detection of HPV in specimens of OLP (%)
O'Flatharta *et al*.[45]	38	PCR	26,3% (16)
Ostwald *et al*.[46]	65	PCR	7,7% (6-11); 9,2% (16-18)
Campisi *et al*.[47]	71	PCR	19,7% (6,16,18,31,33)
Giovannelli *et al*.[48]	49	PCR	24,50% (6,16,18,33,53)
Cianfriglia *et al*.[49]	15	ISH	20% (6-11,31-33-51,16-18)
Khovidhunkit *et al*.[50]	16	PCR	0% (NA)
Szarka *et al*.[51]	119	RT-PCR	32.8% (6,11,16,18,31,33)
Razavi *et al*.[52]	29	PCR	31% (18)
Yildirim *et al*.[40]	65	IHC	21% (16)
Mattilla et al.[53]	82	PCR	15.9% (6,11,16,31,33)

ISH: in situ hybridization; PCR: polymerase chain reaction; RT-PCR: real time polymerase chain reaction; IHC: Immunohistochemistry; In parenthesis: HPV probe positive; NA: Not available.

A positive association between HCV and OLP has been recorded, especially in the populations from the Mediterranean countries, the United States, Saudi Arabia, Taiwan and Nigeria, when compared with other regions in the world (Table 2), suggesting a possible geographic heterogeneity.[74] However, three recent meta-analyses have demonstrated strong evidence between HCV and OLP in all regions of the world.[91-93] This association could be explained by the ability of the HCV virus to infect other cells in addition to hepatocytes, as epidermal cells and the high mutation rate of the virus results in repeated activation of the immune cells, increasing the risk of developing autoimmune diseases.[94] Besides, it is known that in patients with chronic liver disease, the treatment with interferon gamma (INF- γ) may lead to the oral lichenoid reaction to that drug.[95] Furthermore, HCV-associated OLP appears to be a distinct subtype of that disease, since studies have shown an increased frequency of HLA (Human Leukocyte Antigens) class II allele, HLA-DR6 in these cases compared with OLP patients without hepatitis C virus.[95-98] Thus, further studies need to be conducted in order to clarify the role played by the HCV infection in the OLP pathogenesis.

Table 2: The prevalence of HCV in OLP cases published from January 2001 to December 2012 (Pubmed).

Reference	N	Country	Detection of HCV in specimens of LPO (%)
Beaird *et al*.,[57]	24	USA	16,6%
Erkek *et al*.,[58]	54	Turkey	12,9%
Kirtak *et al*.,[59]	73	Turkey	6,8%
Daramola *et al*.,[60]	57	Nigeria	15,7%
Eisen, 2002[14]	195	USA	0%
Figueiredo *et al*.,[61]	68	Brazil	8,8%
Garg *et al*.,[62]	86	Nepal	0%
Mignogna *et al*.,[63]	600	Italy	27,5%
Prabhu *et al*.,[64]	65	India	0%
Gimenez-García and Pérez-Castrillón,[65]	101	Spain	8,9%
Klanrit *et al*.,[66]	60	Thailand	6,6%
Mahboob *et al*.,[67]	184	Pakistan	23,3%
Bokor-Bratic,[68]	48	Serbia and Montenegro	0%
Campisi *et al*.,[69]	859	Italy	27,7%
Chung *et al*.,[70]	32	Taiwan	43,7%
Denli *et al*.,[71]	140	Turkey	5%
de Mattos Camargo *et al*.,[72]	50	Brazil	2%
Ghodsi *et al*.,[73]	146	Iran	4,7%
Harman *et al*.,[74]	128	Turkey	6,2%
Karavelioglu *et al*.,[75]	41	Turkey	4,8%
Lodi *et al*.,[76]	303	Italy	19,1%
Asaad and Samdani,[77]	114	Saudi Arabia	26,3%
Luis-Montoya *et al*.,[78]	36	Mexico	2,7%
Rahnama *et al*.,[79]	66	Iran	1,5%
Das *et al*.,[80]	104	India	1,9%
Khaja *et al*.,[81]	52	India	44%
Ali and Suresh,[82]	40	Saudi Arabia	0%
Giuliani *et al*.,[83]	82	Italy	11,4%
Yarom *et al*.,[84]	62	Israel	4,8%
Stojanevic *et al*.,[85]	173	Slovenia	1,2%
Lin *et al*.,[86]	104	Taiwan	22,1%
Zhou *et al*.,[87]	232	China	1,7%
Konidena and Pavani,[88]	25	India	12%
Nagao and Sata,[89]	59	Japan	67.8%
Jayavelu and Sambandan,[90]	30	India	0%

Regarding the association between OLP and viral infections it can still be pointed out that some cases of this disease have been associated with HIV infection. Nevertheless, the lesions might be more related to antiretroviral therapy than to actually the infectious agent.[99,100]

With respect to the antigen of intrinsic origin, the *heat shock protein* (HSP) stands out, expressed by all cell types,[101] functioning essentially for cell communication, differentiation and growth, signal transduction and apoptosis.[102] The increase in this protein expression can occur in response to several exogenous agents, such as temperature change, medications, viruses, nutrients deprivation and growth factors. This protein high expression in OLP suggests that it might be an auto-antigen of the disease.[103] The identification of an auto-antigen in the OLP lesions associated with the characteristics of the disease, such as chronicity, association with other autoimmune illnesses, preference for adult female patients, presence of lymphocyte T in the lesions, and effectiveness of the immunosuppressive therapy reinforces the autoimmune theory.[104] However, as described above, the HSP super-expression can occur due to several exogenous agents and, therefore, be a retarded event, not the disease trigger.[103,105] In spite of the weak expression of TGF-β1 - protein with immunosuppressive effects -, failure of T cells apoptosis induced by keratinocyte and Langerhans cell maturation, and keratinocyte apoptosis in OLP are other situations that suggest the autoimmune origin for this disease.[22]

Psychological disorders, such as depression, anxiety and stress have been investigated in the OLP etiology**,** as patients with the disease report more frequent development or exacerbation of lesions during periods of greater emotional tension.[106-110] However, studies that have evaluated those disorders in OLP patients have shown controversial results. Some authors found a positive association

between OLP and presence of psychological alterations,[108,111-113] while others did not find that association.[107, 114,115] The use of different inventories, as well as their subjectivity and the lack of methodological standardization of studies are responsible for the controversial results. Moreover, OLP etiopahogenesis is complex and presumably dependent on the interaction of different factors. It is believed that stressful situations could influence the development of the disease, modifying and promoting dysregulation of immune functions with alteration of the balance of Th1/Th2 cytokines and increased Th2 response.[116-119]

The genetic predisposition has been hypothesised in OLP etiology.[120] In this context, many studies have focused on the relationship between HLA and OLP, demonstrating that the HLA-DR1 is frequently associated with cutaneous idiophatic LP, but not in OLP[121] and the HLA-DR6 is usually linked to hepatitis C virus-associated OLP.[95-97] Moreover, Th1/Th2 cytokine polymorphisms have been investigated. Between them IL-18 and IL-4,[122,123] IL-6,[124] TNF-α,[123,125,126] IL-10[126,127] and INF-gama[123,125] seem to have some influence on the susceptibility and progression of OLP. Recently, one mutation in the chromosome 3p14-3q13 has been identified in genetic linkage analysis study, which was suggested as a possible responsible factor for OLP in a Chinese family with five affected individuals.[128] However, it is still too early to say that the disease is genetically determined as there is the need of confirmation by further studies in different geographical areas.[2]

Pathogenesis

Several cell types, proteins of the extra-cellular matrix and chemokines, contribute to the onset of the OLP through the activation of different pathways. The presence of cells that involve migration and activation of T cells and killing of

keratinocytes produce antigen-specific cell-mediated immune response, however, performance of matrix metalloproteinases, chemokines and mast cell are responsible for non-specific immune response.[15,22] Finally, circulating autoantibodies to desmoglein 1 and 3[129] and identification of IgA and IgM,[130] suggest a role of humoral immunity in OLP.

Cells involved in the pathogenesis of oral lichen planus

The cells involved in the OLP pathogenesis are: keratinocytes, $CD8^+$ T lymphocytes (*Suppressor)*, $CD4^+$ T lymphocytes (*Helper*), dendritic cells, mastocyte and macrophages whose levels vary according to the stage in which the lesions are found. In OLP early stages, the helper-T lymphocyte levels, the macrophages and the dendritic cells are higher than the rates presented by more advanced stage lesions, which present high levels of supressor-T lymphocytes.[131] That distribution suggests that in the early stages there is the predominance of antigen-presenting cells, as well as those cells responsible for inducing an inflammatory response, and the predominance of defence cells occurs in more advanced stages, which will determine the keratinocyte apoptosis.[132]

1. Keratinocyte

Keratinocyte, the cell that forms the epithelium of the oral mucosa, is associated with the secretion of type IV collagen and laminin V, proteins that form the basement membrane,[133] which, in turn, is essential for maintaining the keratinocyte vitality. This shows a close relationship and dependence between both structures.[15] In the OLP, the keratinocyte is the target cell, the one to suffer apoptosis,[1,15,104]

however, for this to occur this cell needs to express an antigen - still unknown - in the early stages of the disease development.[15] Once active, those keratinocytes secrete chemokines, which attract lymphocytes and other immune cells that can induce OLP development, as well as favour the disease chronicity[134,135] (Figure 2a).

2. Dendritic Cell (DCs)

DCs present an important role in the immunological response, as they activate T cells through antigenic stimulation.[136] Studies have revealed an increase in the number of DCs in OLP, indicating that they may be associated with its pathogenesis.[137-140]According to Santoro et al.,[138] the increase of different subsets of DCs, such as Langerhans Cells (LCs), stromal DCs and plasmacytoid dendritic cells (PDCs) may promote inflammatory response in OLP. Among these, LCs are the most dendritic cell studied. Those cells reside in the supra-basal layers of the stratified epithelium of the skin and oral mucosa whose function is to capture and present antigens. When these cells capture the antigens, they are activated, migrate to the regional lymphonodes and are introduced to the T lymphocytes, producing a primary immune response. Thus, when the LCs recapture that antigen, it will be recognised by the T lymphocytes circulating memory, which will induce a secondary immune response.[141,142] In the OLP lesions, a high number of LCs are present on the basal layer of the epithelium.[24] It is suspected that in this lesion the LCs play an important role in presenting antigens to the T lymphocyte through class II Major Histocompatibility Complex (MHC) molecules,[143] introducing not only an initial sensitivity to the antigen (primary immune response) but also a subsequent secondary immune response which permits the appearance of the disease clinical signs.[141]

3. CD4$^+$ T Cell

CD4$^+$ T lymphocytes are cells capable of activating the B lymphocytes, macrophages and T CD8$^+$ lymphocyte and thus, responsible for orchestrating different cells during the immune response. These cells are classified into three sub-groups: Th1, Th2 and Th17, morphologically undistinguishable but distinct by cytokine production. Th1 sub-group is characterised by the production of IFN-γ, IL-2 and TNF-α, which are macrophage and cytotoxic T lymphocytes activating cytokines. Th2 sub-group secretes IL-4, IL-5, IL-10 and IL-13, elements that are crucial for the production of antibodies. Th17 sub-group, recently discovered, produces IL-26, IL-22 and IL-17. The latter, an important inducer to inflammatory response, when uncontrolled, is associated with different autoimmune conditions, such as multiple sclerosis, psoriasis and lupus.[144] Recently, the proportion of Th1 and Th17 cells and serum IL-17 levels in patients with OLP were significantly greater than controls, especially in the atrophic-erosive OLP group compared with reticular OLP group, suggesting that Th17 cells and their cytokine Th17 might play an important role in OLP pathogenesis.[145] In the OLP lesions, the CD4$^+$ T cells form most of the lymphocytes present in small clusters deeper down in the subepithelial lymphocyte-rich band[146,147] or in the lamina propria;[23] however, they are not present in the areas of basement membrane rupture.[148] In this disease, the CD4$^+$ T lymphocytes can be activated, at least partially, by antigens associated with class II MHC expressed by LCs or by the keratinocytes. From this point, the CD4$^+$ T lymphocytes activate the cytotoxic T lymphocytes by linking *Request to Cytotoxic Activity* (RCA) receptor with RCA on the surface of CD8$^+$ T lymphocyte or by secreting Th1 derivated citokynes, such as IFN-γ, IL-2 e TNF-α[15] (Figure 2b). In addition, the IFN-γ local production can

maintain the class II MHC expression through keratinocytes, thus contributing to the lesion chronicity.[149]

4. CD8$^+$ T Cell

CD8$^+$ T lymphocytes are cells that recognize antigens presented by the class I MHC molecules determining its elimination by cytotoxity.[144] In the OLP, the T CD8$^+$ cell infiltrate is mainly found in the intra-epithelial region and in the basement membrane disruption areas, adjacent to the basal keratinocytes destruction.[24,148,150] It should be pointed out that the antigen identification, expressed by the basal keratinocytes can occur either through its routine vigilance or attraction, promoted by the chemokines produced by activated keratinocytes.[15] At the end of this process, the caspase cascade occurs via different mechanisms: 1) activation of perforins and granzymes; 2) expression of Fas L receptor (CD95) that interacts with the Fas molecule on the surface of the keratinocytes, and 3) TNF-α secretion, which is linked to TNF-α receptor in the target cell, determining the keratinocyte apoptosis[15] (Figure 2c).

5. Mastocyte Cell (MCs)

Mastocyte Cells are derived from the CD34$^+$ hematopoietic progenitor that have the ability to activate T lymphocyte, suffer degranulation, and release a series of mediators that modulate the inflammatory response.[142] There is an increase in the MCs density in the OLP lesions, which are preferentially located in the lamina propria, near blood vessels and nerves.[151,152] Moreover, MCs density in this disease is markedly higher in the basement membrane rupture sites, suggesting that this cell might play a direct role in the basement membrane destruction, as well as in the T

$CD8^+$ lymphocyte migration to the intra-epithelial region.[148] However, recently, a greater number of mast cells has been identified in deeper connective tissue.[153] Approximately 60% of these mastocytes are found degranulated, releasing cytokines such as IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13 and IL-16, TNF-α, Chymase and RANTES.[15,154,155] It is known that IL-16 stimulates T lymphocyte direct migration through the connective tissue and that the TNF-α can regulate the secretion of adhesion molecule and favor the release of lymphocytes to the extra-vascular region. These lymphocytes, in turn**,** secrete RANTES and other mediators, stimulating the mastocytes to release TNF-α. This proves there is an interaction between mastocytes and T lymphocytes, which, through a cyclic mechanism, might promote OLP chronicity.[22,131] Chymases are proteases capable of inducing MMP-9 production by the T lymphocyte,[156] which proves the indirect action that the mastocytes can have in the destruction of the basement membrane[157] (Figure 2d).

6. Macrophages

Macrophages are phagocyte cells derived from blood monocytes, recruited for the tissues in the presence of chemotaxis signals. They are present in the healthy oral mucosa and in larger numbers during pathological processes.[158] Macrophages are classified in M1 (pro-inflammatory) and M2 (anti-inflammatory) according to the functions of their effectors.[159] The M1 macrophages might exacerbate OLP manifestation through the production of pro-inflammatory agents such as TNF-α e IL-1β. Those agents, in turn, regulate the presence of adhesion molecules on the endothelial cells surface inducing the production of chemokines (RANTES) by the keratinocytes, resulting in an increase in the inflammatory cells recruitment inside the lesion. Furthermore, it must be pointed out that the production of TNF-α by the

macrophages can initiate the basal keratinocyte apoptosis and, indirectly, increase the disruption rate of the basement membrane by MMP-9, produced by T cells[158] (Figure 2d).

Chemokines involved in the pathogenesis of oral lichen planus

Chemokines form a family of little cytokines, initially identified by their modulator action on the inflammatory response. More attention has been given to these proteins nowadays, especially due to their function on the endothelial cells.[160] Besides, recent evidence has shown the role chemokines play on different autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis.[161] One of the most widely studied chemokines is RANTES (Regulated on Activation Normal T-cell Expressed and Secreted), a member of the CC chemokine family, produced by different cell types, including activated T lymphocyte, keratinocytes and mastocytes. Its biological effect, especially the recruitment of lymphocyte, natural Killer cells and mastocytes, is produced when RANTES is linked to different receptors, such as CCR-1, CCR-3, CCR-4, CCR-5, CCR-9 and CCR-10, found on the cell surface.[15] It has been proved, *in vitro*, that RANTES production by the T cells initiates mastocyte degranulation, which liberates TNF-α and chymase and, in turn, stimulates the release of RANTES by the cells, attracting more mastocytes and their consequent degranulation. Therefore, this cyclical mechanism can contribute to the OLP lesions chronicity.[15,25,155]

Matrix metalloproteinases (MMPs) involved in the pathogenesis of oral lichen planus

The MMPs form a family of zinc-dependent proteases, with at least 20 members involved in cell migration, angiogenesis and proteolytic activation of growth factors, events necessary for the normal tissue repair as well as in wound healing and tumoural invasion.[162] Their function is in part regulated by tissue inhibitor of metalloproteinases (TIMPs), among which, the most well known are TIMP-1, TIMP-2 and TIMP-3. An imbalance between MMPs and TIMPs can be associated with the tissue destruction seen in some pathologies, like cancer, arthritis and cardio-vascular diseases.[163,164] The MMPs that come from T-cells take part in the movement of those cells in extravascular tissues and in their migration through the basement membrane.[15] Studies have identified a difference in the MMP-2 expression and distribution in the OLP. Sutinen et al.[165] verified that the MMP-1 expression, besides being low, was restricted to fibroblasts of the sub-epithelial region, while MMP-2 was not detected in the 10 samples studied. Zhou et al.[157] found that MMP-2 and MMP-3 were mainly expressed in the epithelial region, and MMP-9 was identified in the sub-epithelial inflammatory infiltrate. Still, according to those authors, MMP-9 induces the rupture of collagen IV, and thus might be associated with the basement membrane degradation and facilitate the T-cell intra-epithelial degradation. In a Rubaci et al.[166] study the expression of MMP-2 and MMP-7 in epithelium and connective tissues from OLP lesions were greater than normal oral mucosa. Likewise, the MMP-2/TIMP-1 and MMP-7/TIMP-1 ratios were higher in the OLP patient group than in the control group. These results suggest that increased MMPs expression and imbalance between MMPs and TIMPs play a role in the pathology of OLP. It has been pointed

out that different OLP clinical forms (erosive and non-erosive) are associated with significant differences in the MMPs expression levels, especially MMP-1, MMP-2, MMP-3 and MMP-4, which seem to be more associated with the development of erosive lesions.[167,168]

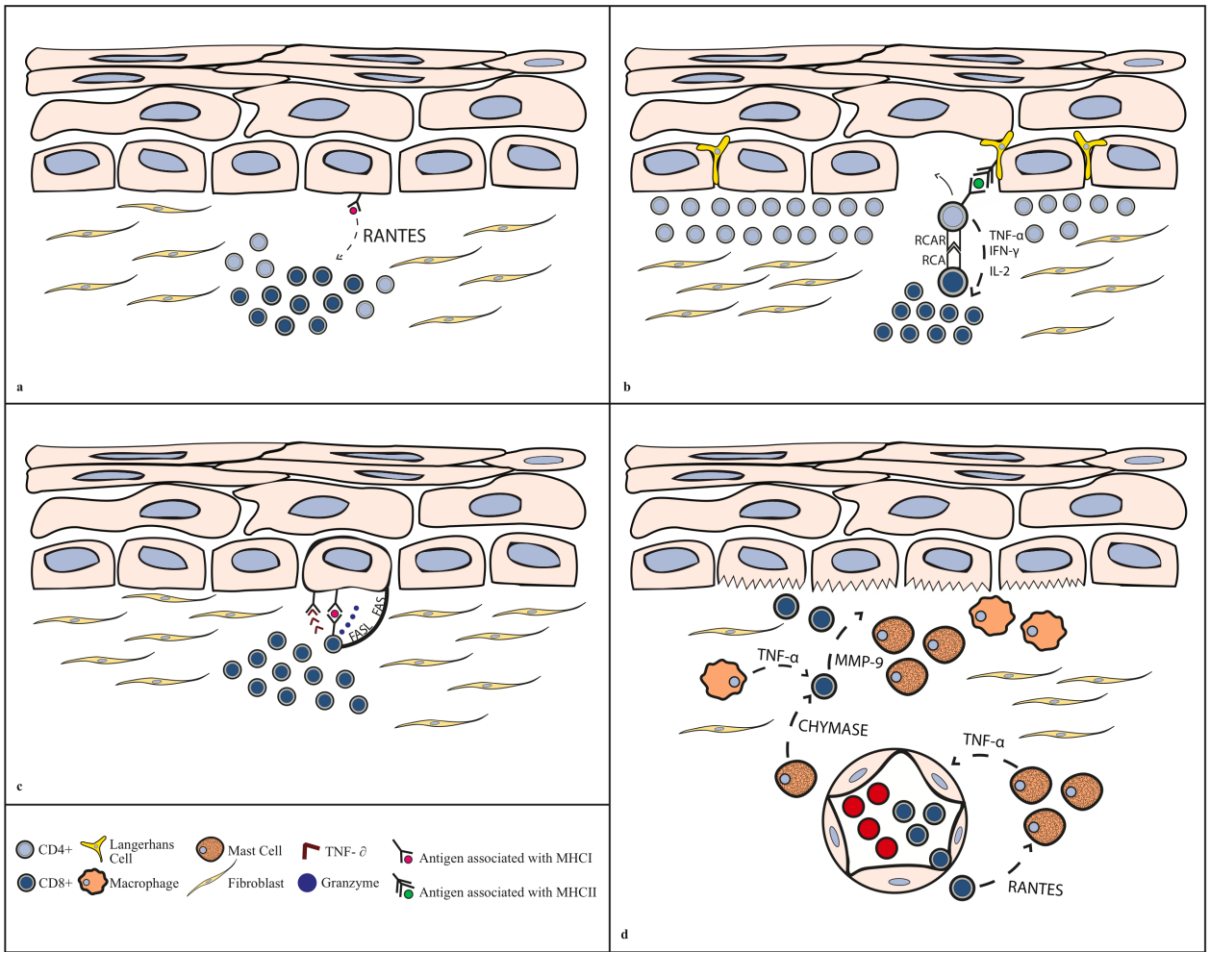

Figure 2: (a): Keratinocytes activated by antigens associated with class I MHC secrete chemokines, which attract lymphocytes; (b): T CD4+ lymphocytes activated by antigens associated with class II MHC expressed by LCs activate the cytotoxic T lymphocytes; (c): Apoptosis of keratinocytes via differents mechanisms; (d): Interaction between mastocytes and T lymphocytes which, through a cyclic mechanism, might promote OLP chronicity. Chymases inducing MMP-9 production by the T lymphocyte, which proves the indirect action that the mastocytes can have in the destruction of the basement membrane.

Role of Immune Cells and their cytokines in malignant transformation of OLP

Although the causes for the malignant transformation in OLP patients have not been totally clarified, it is believed that nutritional disorders, genetic factors and immunosupression induced by certain therapies used in OLP treatment, as well as the action of some external mutagenic agents (tobacco, alcohol, candidiasis and HPV virus) could make the oral mucosa more sensitive, increasing malignancy risk.[15,120]

Besides, the evidence has shown a close relationship between chronic inflammation and tumoural growth and development.[169,170] Several chemical mediators are released during the chronic inflammatory process that in the long term can influence the expression of genes that control proliferation and apoptosis, thus promoting carcinogenesis.[158,169,171]

Among the main pro-inflammatory cytokines involved in this process, those derived from TAM (tumor-associated macrophages) and Th17, such as TNF, IL-1β, IL-6, IL-12, IL-23, stand out. They can activate transcription factors, like AP-1 (Activator Protein), NFKB (Nuclear Factor KB) and STAT-3 (Signal Transducer and Activator of Transcription 3), which promote the expression of many other mediators with pro-inflammatory, pro-angiogenic and immunoregulatory activities thus performing an important role in this disease malignisation.[158,169,171] However, the role of some cytokines can vary according to the intensity of their expression[15] and tumour development stage.[163] The TGF-β can inhibit tumor growth in early carcinogenesis stages but, on the other hand, it can favor neoplasia growth especially for inducing angiogenesis and MMP-9 expression in more advanced stages.[172] Other inflammation mediators like chemokines and their receptors have

been investigated in tumor initiation and promotion phases.[173] Molecular studies have shown that RANTES can induce the expression of important cell enzymes such as phosphatidylinositol (PI) 3-Kinase and Akt/protein kinase B, which can induce pro-proliferation signals that influence cell survival and malignant transformation.[174,175]

In addition, an imbalance between MMPs and TIMPs can be associated with the OLP malignant transformation, being MMP-2 and MMP-9 a possible marker of the malignant transformation potential of the disease.[166,168]

Chronic inflammation can also induce cyclooxygenase (COX) release, an enzyme that transforms arachidonic acid into prostaglandin; its COX-2 isoform, when super-expressed, has been associated with important carcinogenesis stages[176] such as angiogenesis[177] and apoptosis.[178] A COX-2 super-expression has been identified in the OLP, so it was suggested that it could be associated with the increase of its malignant transformation potential.[179]

More recently, oxidative stress increase and imbalance in the antioxidant defense system have been found in the OLP patients oral fluids.[180-182] Battino et al.[180] have found low levels of saliva uric acid and an increase in serum gamma glutamyl transpherase (GGT) and total antioxidant capacity of saliva in patients with OLP compared with control patients. Similarly, Ergun et al.[181] showed that, total antioxidant defense (TAA) serum in OLP patients was significantly lower than that in healthy subjects and that salivary lipid peroxidation product malondialdehyde (MDA) levels were significantly higher in the OLP group compared with control group. Results of Kawanishi et al.[182] revealed that nitrative and oxidative DNA lesion products were expressed in epithelial cells and inflammatory cells at the carcinogenesis site in human and animal models. Thus, it suggests that those

elements contribute to the development of DNA damage and malignant transformation of inflammation-associated carcionogenesis.

Finally, cytokines from T $CD4^+$ lymphocytes, like IFN-γ, TNF-α and IL-12, and the cytotoxic activity of lymphocyte T $CD8^+$ also present in the chronic inflammatory response play an important role in the inhibition and death of malignant cells. Thus, the appearance of malignant phenotypes in OLP patients can be associated with an imbalance between the activity of different kinds of cells and the expression of different inflammation mediators, inhibitors and carcinogenesis promoters.

Conclusion

Despite the extensive literature regarding the OLP origin and development mechanism, its etiology remains uncertain and the pathogenesis is still the object of much speculation. One believes that different external agents, especially virus, and internal agents, like stress, and the HSP antigen expression, associated or not, can trigger OLP. Subsequently**,** lymphocytes, the main cell to form OLP lesions, produce and respond to a great range of inflammatory mediators and cytokines that can affect the keratinocytes and stimulate their apoptosis, determining the clinical onset of the disease.

It remains uncertain, in spite of much discussion, whether OLP has the potential for intrinsic malignant transformation, or if external factors are associated with its malignisation process. Since carcinogenesis is a complex process and presents multifactorial origin, it is believed that there may be a synergism between intrinsic (inflammation mediators) and extrinsic agents (tobacco, alcohol, viral infections) for the OLP malignant transformation to occur.

Besides, it is believed that Th17, recently discovered, should be further investigated in OLP lesions, once it has been identified in different autoimmune diseases and is associated with protein production that can be involved in the malignisation of chronic inflammatory processes. Furthermore, studies aiming at elucidating the role of oxidative stress and other cytokines, especially, TGF-β, chemokines and MMPs in the OLP malignant transformation must be carried out, as they perform important functions associated with chronicity and disease aggressiveness.

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4 ARTIGO DE PESQUISA

IMMUNODETECTION OF VEGF AND ANGIOPOIETINS 1 AND 2 IN ORAL LICHEN PLANUS

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IMMUNODETECTION OF VEGF AND ANGIOPOIETINS 1 AND 2 IN ORAL LICHEN PLANUS

ANGIOGENESIS IN ORAL LICHEN PLANUS

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Abstract

The immunodetection of VEGF and angiopoietins (ANG) 1 and 2, as well as the number of blood vessels was investigated in reticular (ROLP) (n=21) and atrophic-erosive (AEOLP) (n=11) oral lichen planus, as well as in oral fibroepithelial hyperplasia (OFH) (n=10), epithelial dysplasia (OED) (n=10) and squamous cell carcinoma (OSCC) (n=10). There was no significant difference in the immunodetection of ANG-1 and ANG-2, nor in the number of blood vessels between OLP groups. The ROLP group showed significantly greater immunodetection of VEGF compared with the AEOLP group. The comparison between the OLP group and each control group showed significantly greater levels of ANG-1 in the OLP group compared to the OFH group and no significant difference in ANG-2. VEGF levels in the OLP group were significantly higher than in the OFH group and significantly lower compared to the OSCC group. The results indicate that angiogenesis in OLP can not be associated to the different clinical forms of this disease. The lack of correlation between the markers in the different groups indicates the involvement of other pro-angiogenic agents in the neoformation vascular and reflects the complexity this process. Immunodetection of these growth factors in the OLP and OED groups was similar, suggesting that angiogenesis in OLP may behave similarly to the epithelial dysplastic lesion.

Introduction

Angiogenesis, the process by which new vessels are formed from pre-existing vascular structures, is controlled by numerous growth factors and pro-angiogenic cytokines, as well as by various endogenous inhibitors of neovascularization.[1,2] Under physiological situations, these activators and inhibitors are in balance, but in

pathological situations there can be persistent or excessive activation of pro-angiogenic agents.[1,3]

Vascular endothelial growth factor (VEGF) is one of the most potent and known stimulators of angiogenesis, showing the ability to induce vascular permeability,[4] besides the proliferation and migration of endothelial cells, essential steps for the development of new blood vessels.[5,6] Other growth factors essential for vascular formation are the angiopoietins (ANG), among which ANG-1 and ANG-2 are the most known and best characterized.[7] In contrast to VEGF, angiopoietins are angiogenic mediators whose action does not induce mitotic activity in endothelial cells.[8] Despite their biological activities not being completely elucidated, it is known that ANG-1 is constantly secreted in situations of vascular quiescence, as well as during the process of vascular maturation, a later step in angiogenesis.[8-10] Besides, ANG-1 acts as an anti-inflammatory cytokine.[7] On the other hand, ANG-2, despite showing affinity for the same Tie-2 receptor of ANG-1, does not phosphorylate it, thus exerting an antagonistic effect of ANG-1,[7,11] destabilizing the vascular endothelium, which causes the regression of neoformed vessels and apoptosis in endothelial cells.[12] However, some studies have identified ANG-2 as an agonist for the Tie-2 receptor, inducing angiogenesis and the inflammatory response,[9] when this receptor is expressed in non-endothelial cells[11] or in the presence of VEGF.[13]

In chronic inflammatory diseases such as psoriasis,[14] rheumatoid arthritis[8,15] and bowel diseases,[16] investigations have revealed a strict relation between angiogenesis and disease activity, which contributes to its persistence and chronicity. In addition, it has been demonstrated that angiogenesis it related to cell proliferation[17] and can be an early event in malignant transformation.[18-20]

Oral lichen planus (OLP) is a chronic inflammatory disease of unknown etiology and uncertain pathogenesis[21] and although its risk of malignant transformation is low,[22-24] it is considered a potentially malignant disease.[25] Few studies have investigated angiogenesis in OLP,[26-29] but it is believed that the rich vascularization present in the lesions probably occurs as a result of hypoxia, caused by the proliferation of lymphocytes.[30] Recent studies have demonstrated an increase in the expression of VEGF[29] and in microvessel density (MVD)[26,28] in OLP, especially in the erosive form, suggesting that angiogenesis can play a distinct role in the different clinical forms of this disease.[26]

In this study, the immunodetection of VEGF, ANG-1 and ANG-2, as well as the number of blood vessels was investigated in specimens of reticular and atrophic-erosive OLP. Besides, these markers were determined in specimens of oral fibroepithelial hyperplasia, oral epithelial dysplasia and oral squamous cell carcinoma and compared with values present in OLP, in order to find a relation between those angiogenic growth factors and biological behavior.

Materials and Methods

This study was approved by the Committee of Ethics in Research of the Pontifical Catholic University of Rio Grande do Sul (PUCRS), under protocol 11/05540.

Tissue samples

Specimens of biopsies embedded in paraffin were obtained from adult patients of both sexes. According to the clinical and histopathological diagnosis, based on

WHO[30] criteria, the sample was distributed into the following groups: OLP, 32 specimens of oral lichen planus, subdivided into ROLP with 21 specimens of reticular oral lichen planus and AEOLP with 11 specimens of atrophic or erosive oral lichen planus; OFH, 10 specimens of oral fibroepithelial hyperplasia; OED, 10 specimens of oral epithelial dysplasia; and OSCC, 10 specimens of oral squamous cell carcinoma. The inclusion criteria utilized for the selection of cases of ROLP were the presence of reticular, plaque or papule patterns and, on microscopic examination, inflammatory infiltrate composed mainly of lymphcytes, arranged in a band in the subepithelial region, signs of hydropic degeneration in the basal layer and absence of epithelial dysplasia. In the diagnosis of AEOLP, the clinical requirement was the presence of erosive or atrophic types and histopathological evidence of atrophic or ulcerated epithelium, besides the above characteristics. When there was an association between white and atrophic/erosive lesions, they were inserted in the AEOLP group, since these areas were included in biopsies. The lesions of the OFH group were required to microscopically show fibroplasia, hyperplasia of epithelial lining, and also lymphoplasmocytic infiltrate and vascularization in the lamina propria. The cases of OED were included when there was clinical manifestation of leukoplakia, erythroplakia or erythroleukoplakia and microscopic evidence of epithelial dysplasia. Inclusion in the OSCC group required epithelial neoplastic lesions to appear moderately differentiated. Biopsy specimens in which it was not possible to perform all steps in the immunohistochemical assay were excluded. The medical charts were analyzed and data such as gender, age and localization were recorded, even as clinical pattern and number of sites affected, in the cases of OLP.

Immunohistochemistry

Three new sections were obtained from each sample, with a thickness of 3 µm, which were deparaffinized in xylene and rehydrated in alcohol. Endogenous peroxidase was blocked by immersing the sections in 0.3% hydrogen peroxide in methanol. Antigen retrieval was done with citrate buffer, pH 6.0 (Dako Corporation, Carpinteria, CA, USA) in a steamer for 20 minutes. Next, the sections were cooled to ambient temperature. The slides were then incubated in an oven at 30°C for 1 hour with the primary antibodies anti-VEGF (1:200; monoclonal antibody, anti-human, Santa Cruz Biotechnology, CA, USA), anti-ANG-1 (1:100; polyclonal antibody, anti-human, R&D System, Minneapolis, MN, USA) and anti-ANG-2 (1:50; polyclonal antibody, anti-human, R&D System, Minneapolis, MN, USA). Primary antibodies were revealed using the avidin-biotin system (Dako Corporation, Carpinteria, CA, USA) with 45 minutes incubation at 30°C. The sections were counterstained with Harris' hematoxylin and mounted with Entellan (Merck KgaA, Darmstadt, Germany). Amygdala was used as a positive control for VEGF and prostate was used for ANG-1 and ANG-2. The primary antibody was omitted in the negative control.

Immunodetection of VEGF, ANG-1 and ANG-2

The immunodetection of the growth factors evaluated was visualized by the presence of a brown color in the tissues. When immunodetection was positive, the structures evaluated were epithelial tissue, connective tissue with its cellular elements (fibroblasts and inflammatory cells) and blood vessels.

The quantitative determination of the antigens VEGF, ANG-1 and ANG-2 was carried out by a single blinded observer, in ten microscopic fields captured at

equidistant points on each slide at 200x magnification. A Zeiss Axioskop 40 light microscope (Zeiss; Oberkochen, Germany) was used coupled to a Roper Scientific videocamera (Media Cybernetics, Silver Springs, MD, USA) and a Pentium IV 2.2 GHz computer with 512 MB. The images captured were later transferred to the software Image J for Mac, version 1.47a (CyberMedia, NY, USA). In each image, the percentage of area showing positive staining was calculated by the semi-automated segmentation technique. The number of blood vessels was determined in slides immunostained for ANG-1, utilizing the same software, by means of a manual counting tool. Structures in which the presence of endothelium was evident were considered vessels.

Statistical analysis

The data were processed and analyzed using the statistical software SPSS for Windows, version 17.0. P-values ≤0.05 were considered significant. The data were initially analyzed by means of descriptive statistics. Student's t-test for independent samples was applied for comparison of immunodetection of VEGF between the NEOLP and EOLP groups. This test was also utilized to compare the immunodetection of VEGF between the OLP group and each of the control groups. Comparison of expression of ANG-1 and ANG-2 between the ROLP and AEOLP groups was carried out using the non-parametric Mann-Whitney test, which was also employed for comparing the OLP group with each of the control groups. The comparison of the number of vessels between the ROLP and AEOLP groups was done using the non-parametric Mann-Whitney test. The correlation between the

expression levels of VEGF, ANG-1 and ANG-2 in different lesions studied was evaluated utilizing the Spearman Correlation coefficient.

Results

Sample Characterization

The mean age of the ROLP group was 51.44±11.91 years and of the AEOLP group 50.60±10.51 years. Females predominated in the ROLP and AEOLP groups, corresponding to 14 (66.66%) and 8 (72.72%) cases, respectively. In the ROLP group, 9 (42.85%) cases showed the presence of more than one clinical pattern, with a predominance of the reticular pattern, alone or combined in 15 (71.42%). With respect to the AEOLP group, 9 (81.81%) patients also displayed more than one clinical pattern. The reticular pattern was present associated with atrophic-erosive lesions in 7 (63.63%) cases. In regard to localization, both OLP groups exhibited a predominance of lesions at more than one site and the most affected was the buccal mucosa, which corresponded to 11 (52.38%) cases in the ROLP group and 9 (81.81%) cases in the AEOLP group.

In relation to the control groups, it was noted that the OFH group showed a mean age of 58±11.91 years, the OED group 53.44±16.23 years, and the OSCC group 56.8±15.23 years. Among these, the OFH and OED groups showed a predominance of female patients, with 6 (60%) and 8 (80%) cases, respectively. In the OSCC group, there was a predominance of males, corresponding to 8 (80%) cases.

VEGF, ANG-1 and ANG-2 immunodetection

Immunodetection of VEGF and ANG-2 was observed in epithelial, endothelial and connective tissue cells. On the other hand, ANG-1 was detected mainly in epithelial and endothelial cells, while there was discrete staining of fibroblasts (Figures 1, 2 and 3). With regard to the type of staining present in the epithelium, immunodetection of ANG-1 was observed mainly in the membrane and cytoplasm (Figure 1). ANG-2 and VEGF immunostaining was predominantly localized in the cytoplasm and nucleus (Figures 2 and 3) in all the groups studied. In connective tissue, immunodetection of VEGF and ANG-2 was observed in fibroblasts and inflammatory cells in all cases. In relation to the percentages of immunodetection, values of ANG-1 were lower compared to the VEGF and ANG-2 in all the groups.

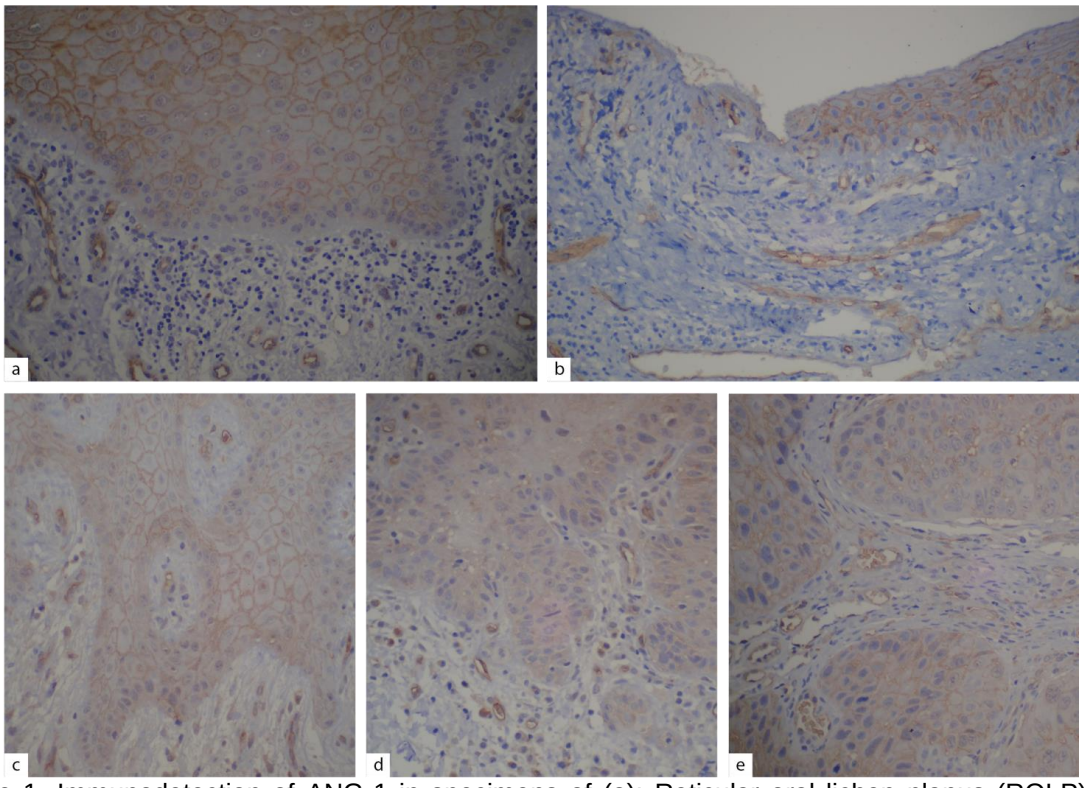

Figure 1. Immunodetection of ANG-1 in specimens of (a): Reticular oral lichen planus (ROLP); (b): Atrophic-Erosive oral lichen planus (AEOLP); (c): Oral fibroepithelial hyperplasia (OFH); (d): Oral epithelial dysplasia (OED); (e): Oral squamous cell carcinoma (OSCC). (Original magnification, x200)

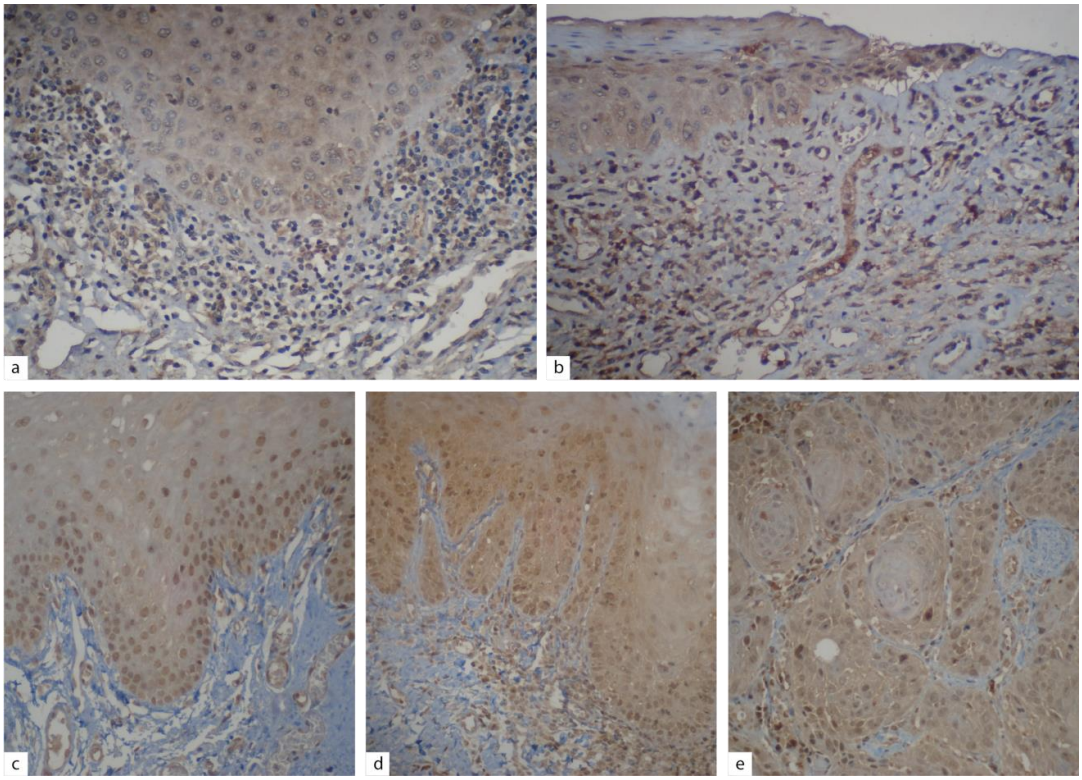

Figure 2. Immunodetection of ANG-2 in specimens of (a): Reticular oral lichen planus (ROLP); (b): Atrophic-Erosive oral lichen planus (EOLP); (c): Oral fibroepithelial hyperplasia (OFH); (d): Oral epithelial dysplasia (OED); (e): Oral squamous cell carcinoma (OSCC). (Original magnification, x200)

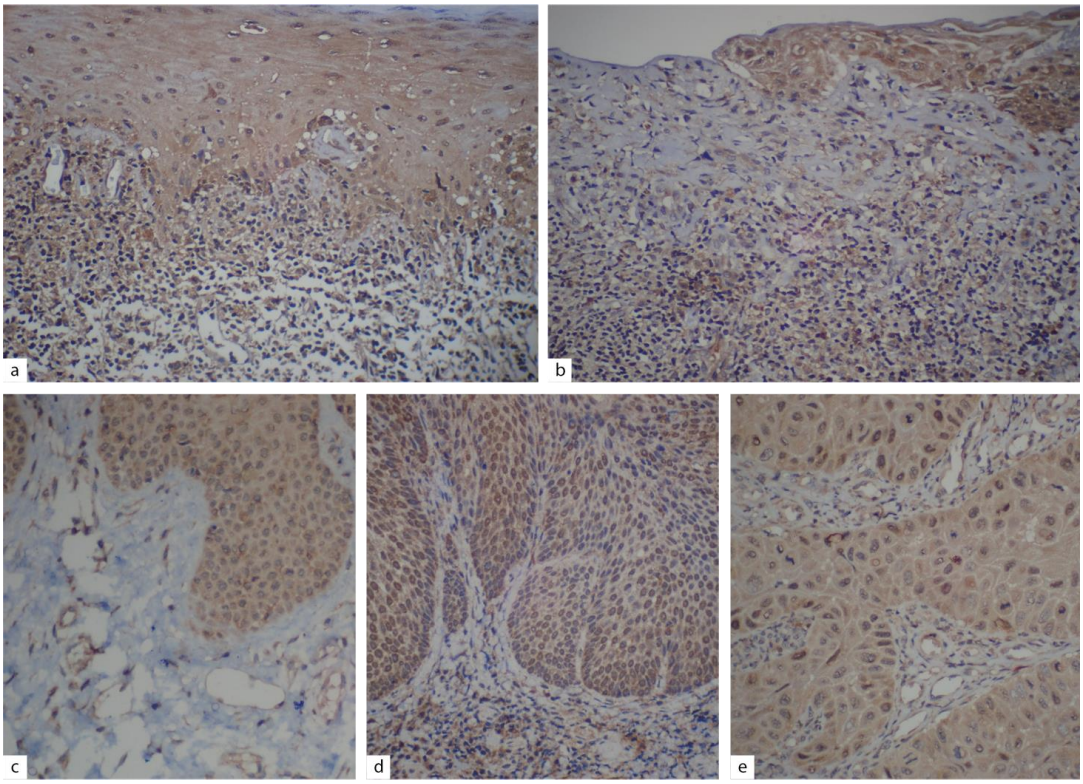

Figure 3. Immunodetection of VEGF in specimens of (a): Reticular oral lichen planus (NEOLP); (b): Atrophic-Erosive oral lichen planus (AEOLP); (c): Oral fibroepithelial hyperplasia (OFH); (d): Oral epithelial dysplasia (OED); (e): Oral squamous cell carcinoma (OSCC). (Original magnification, x200)

Immunodetection of VEGF, ANG-1 and ANG-2 in the ROLP and AEOLP groups is described in Table 1. The ROLP group showed a significantly higher percentage of immunodetection for VEGF (P=0.01) compared to the AEOLP group, while there was no significant difference between the two groups with respect to ANG-1 and ANG-2.

Table 1. Mean and standard deviation of the percentage of immunodetection of VEGF and median and interquartile range of the percentage of immunodetection of ANG-1 e ANG-2 in reticular oral lichen planus (NEOLP) and atrophic-erosive oral lichen planus (EOLP) groups.

	Groups		
	ROLP	**AEOLP**	**P**
VEGF	46.15 ± 16.67	28.95 ± 17.46	0.010[a]
ANG-1	11.61 (6.61-24.05)	4.55 (3.86-23.61)	0.148 [b]
ANG-2	21.97 (10.61-38.19)	27.05 (12.46-57.61)	0.372 [b]

[a]Student T-Test for significance, P-value ≤ 0.05. [b]Mann-Whitney Test for significance, P-value ≤ 0.05.

In relation to the number of blood vessels, the comparison between the ROLP and AEOLP groups did not demonstrate a significant difference (P=0.393) (Figure 4).

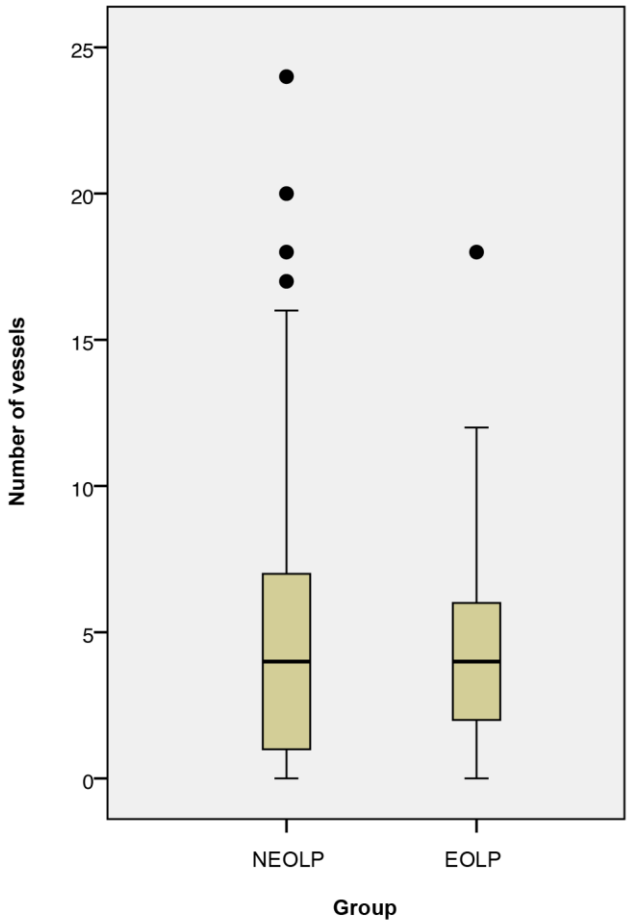

Figure 4. Median and interquatile range of number of blood vessels in reticular oral lichen planus (ROLP) and atrophic-Erosive oral lichen planus (AEOLP) groups.

For the comparative analysis between oral lichen planus and control groups, 32 cases of this lesion were considered, which formed the OLP group. When the immunodetection of VEGF was compared between OLP group and control groups, the OLP group showed a significantly higher percentage than the OFH group (P=0.003) and significantly lower value in relation to the OSCC group (P=0.032) (Table 2).

Table 2. Mean and standard deviation of percentage of immunodetection of VEGF in oral lichen planus (OLP) group and control groups.

OLP (Mean ± SD)	**Control groups**	**Mean ± SD**	**P**
40.23 ± 18.61	**Hyperplasia (OFH)**	19.29 ± 18.56	0.003*
	Dysplasia (OED)	48.44 ± 18.80	0.232
	Carcinoma (OSCC)	56.63 ± 25.52	0.032*

*Student T-Test for significance, P-value ≤ 0.05.

In comparing the immunodetection of ANG-1 between the OLP group and control groups, significantly higher percentages were identified in the OLP group in relation to the OFH group (P=0.043). On the other hand, no significant difference was observed in the immunodetection of this marker between OLP group and the control groups OED and OSCC (Table 3).

Immunodetection of ANG-2 did not demonstrate a significant difference between the OLP group and the different control groups. However, higher percentages were found in the OFH group, lower levels in the OSCC group, and very similar values between the OLP and OED groups (Table 3).

Table 3. Median and interquartile range of the percentage of immunodetection of ANG-1 e ANG-2 in oral lichen planus (OLP) group and control groups.

		Median (p25-p75)	**Control groups**	**Median (p25-p75)**	**P**
ANG-1	**OLP**	10.90 (4,54-23,85)	**Hyperplasia (OFH)**	3.59 (1.53-14.98)	0.043*
			Dysplasia (OED)	7.63 (5.46-18.13)	0.575
			Carcinoma (OSCC)	5.56 (3.76-9.14)	0.104
ANG-2	**OLP**	25.62 (11,34-38,90)	**Hyperplasia (OFH)**	33.55 (17.13-48.17)	0.256
			Dysplasia (OED)	25.50 (11.26-29.40)	0.756
			Carcinoma (OSCC)	16.88 (11.92-32.92)	0.535

*Mann-Whitney Test for significance, P-value ≤ 0.05.

The results did not show any correlation between the immunodetection of VEGF, ANG-1 and ANG-2 in the different groups studied (data not shown).

Discussion

Few studies have investigated angiogenesis in lesions of OLP, and these are limited to evaluations of VEGF, MVD and the adhesion molecules VCAM-1 (Vacular Cell Adesion Molecule-1) and ICAM-1 (Intercellular Adhesion Molecule-1).[26-29] In the present study, angiogenesis was investigated in OLP by the immunodetection of angiogenic agents that act in different steps in the process of vascular neoformation. VEGF induces an increase in vascular permeability[4] and endothelial proliferation, and it is essential in the initial steps of vascular formation.[6] ANG-1 performs functions related to vascular quiescence, but also to the process of vascular maturation, a later step in the process of the formation of new blood vessels.[9,10] ANG-2, in turn, destabilizes blood vessels and, depending on the presence or absence of VEGF, induces vascular regression of favors endothelial cell migration, a step also essential for the initiation of angiogenesis.[9] In addition, this study included as control groups oral fibroepithelial hyperplasia, oral epithelial dysplasia and oral squamous cell carcinoma to establish a comparison of angiogenesis in OLP in relation to these lesions, which show different degrees of aggressiveness. We decided to subdivide the OLP group into the reticular and atrophic-erosive forms, since previous studies showed differences in vascularization,[26,28,29] as well as in proliferative activity,[32] between these clinical forms, which could be a reflection of the more aggressive behavior of the erosive pattern of the disease, suggested by some authors.[33,34] However, since there were no significant differences between the ROLP and AEOLP groups in the immunodetection of ANG-1 and ANG-2 and on the basis of the results

obtained in relation to VEGF, we decided to form a single OLP group to perform comparative analyses between this disease and control groups.

The characteristics of the sample in the different OLP groups were similar to those of previous studies,[22,35,36] where there was predominance of female patients of about 50 years old, who developed mainly reticular lesions localized in the buccal mucosa. These characteristics allowed us to demonstrate that the lesion exhibits a well-established profile that can be identified even in small samples.

In the present study, VEGF was detected in epithelial tissue, as well as in different cells of connective tissue, confirming that the expression of this pro-angiogenic agent occurs in different cell types such as keratinocytes, fibroblasts, activated macrophages and inflammatory cells.[6] The literature reports that ANG-1 is expressed in pericytes, smooth muscle cells, fibroblasts and tumor cells and ANG-2 almost exclusively in endothelial and perivascular cells.[9] However, ours results demonstrated that these growth factors are expressed in epithelium and that ANG-2 is also present in connective tissue cells. Chien et al.[37] demonstrated that in specimens of oral squamous cell carcinomas, these markers were predominantly found in the cytoplasm of tumor cells. In chronic inflammatory diseases such as rheumatoid arthritis, the majority of studies were performed by evaluating the serum levels of ANG-1 and ANG-2,[8,15] thus making it difficult to compare with the type of staining found in our study.

Considering the immunodetection of VEGF, our results revealed that the ROLP group showed significantly higher values in comparison to the AEOLP group. This result is in contrast with that of earlier studies. Tao et al.[26] did not find significant differences in the immunodetection of VEGF between reticular and erosive OLP. On the other hand, Mardani et al.[28] reported significantly higher serum VEGF levels in

patients with erosive versus reticular OLP. Since VEGF is the principal inducer of angiogenesis and considering that atrophic-erosive lesions represent acute processes of the disease, we expected to see higher expression of this growth factor in the AEOLP group. We believe that the significantly lower values in the atrophic-erosive lesions can be associated with the use of topical corticosteroids for the pain caused by the lesions. The utilization of these drugs modulates the inflammatory response, interfering with the expression of VEGF. Rhodus et al.[38] demonstrated a significant reduction in salivary levels of TNF-α, IL-1-α, IL-6 and IL-8 after therapeutic intervention with corticosteroid in erosive lesions of OLP, which influences VEGF levels.[39] Still regarding the immunodetection of VEGF, it should be noted that the value found in the OLP group was significantly lower than in the OSCC group, significantly higher than in the OFH group and not differing in relation to the OED group, suggesting that angiogenesis in OLP can behave in a similar way as more aggressive lesions. Thus, OLP presents a distinct biological behavior compared to other benign chronic inflammatory processes such as fibroepithelial hyperplasia.

There are no reports in the literature about immunodetection of ANG-1 and ANG-2 in lesions of OLP. In this study, the values of these markers did not differ between ROLP and AEOLP groups. The OLP group showed a significantly higher percentage of immunodetection for ANG-1 in relation to the OFH group. The immunodetection of ANG-2 did not significantly differ between the OLP group and the controls, but very similar values were found in the OLP and OED groups. Comparative studies evaluating the expression of proteins such as p53 and bcl-2[40] and cell proliferative activity, by investigating PCNA,[41] also reported similar values between lesions of OLP and epithelial dysplasias.

This study has demonstrated a lack of correlation between Ang-1, ANG-2 and

VEGF in the different groups studied. This finding may be result of the small sample size, but also may reflect the complexity of angiogenesis itself, which can be influenced by a series of other pro-angiogenic cytokines and cell types to determine vascular neoformation.

The comparison of the number of vessels did not demonstrate a significant difference between the ROLP and AEOLP groups. Tao et al.[26] demonstrated significantly higher values MVD of erosive OLP lesions compared with the lesions of reticular OLP, suggesting that angiogenesis is correlated with different clinical forms of this disease, but this notion was not supported by our study. In the present research, we chose not to compare the number of vessels between OLP group and control groups, because they involve lesions of different nature, among which some show a histological field with a predominance of epithelial cells while others a predominance of connective tissue, which could have interfered with the reliability of this analysis.

In conclusion, the results of immunodetection of VEGF, ANG-1 and ANG-2 between ROLP and AEOLP groups indicate that angiogenesis in OLP can not be associated to the different clinical forms of this disease. The lack of correlation between the markers in the different groups indicates the involvement of other pro-angiogenic agents in the neoformation vascular and reflects the complexity of this process. Furthermore, immunodetection of growth factors evaluated was similar between OLP and DEO, demonstrating that angiogenesis in OLP may behave similarly to oral epithelial dysplasia and that this disease has different biological behavior of other chronic inflammatory processes benign as fibroepithelial hyperplasia. Further studies are needed to investigate other pro-angiogenic cytokines to better understand the process of angiogenesis in this disease.

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5 DISCUSSÃO GERAL

O LPO é uma doença inflamatória crônica que desperta grande interesse em pesquisa, visto que permanecem incertezas a respeito de sua origem, desenvolvimento[6,7,13,14] e comportamento biológico.[17,19,20]

Estudos que tentaram desvendar a sua etiologia não conseguiram determinar o antígeno que desencadeia esta doença, nem se existe o envolvimento de um ou mais deles.[13] Uma associação entre o vírus HPV e o LPO tem sido observada,[52-54] o que pode sugerir não somente sua relação com a etiologia, mas também com o potencial de transformação maligna da doença. Por outro lado, isso pode refletir uma maior suscetibilidade das lesões à infecção[55] ou estar relacionado ao tratamento com corticosteróides, que favorecem a replicação viral.[55,56] Outro vírus observado em pacientes com LPO é o HCV,[57-59] muito embora ainda não se entenda totalmente essa associação.[60] Ainda com relação à sua etiologia, apesar de estudos que avaliaram ansiedade, depressão e situações de estresse em pacientes com LPO encontrarem resultados controversos,[10,61-63] observa-se que pacientes com esta doença, geralmente, apresentam desordens emocionais. Portanto, acredita-se que os resultados controversos ocorram por falta de padronização metodológica dos estudos e que alterações emocionais sejam fatores que possam contribuir na etiopatogenia do LPO.

Pesquisas apontam que diferentes mecanismos podem ser ativados durante o processo de desenvolvimento do LPO. Estes são representados, principalmente, pela resposta imune celular antígeno-específica e não específica,[13,64] que determina a apoptose dos queratinócitos. No entanto, devido à complexidade destes processos, envolvimento de diferentes tipos celulares,[65-68] de proteínas da matriz

extracelular[69,70] e de quimiocinas,[71] bem como devido à possibilidade de ocorrer também uma resposta imune humoral,[72,73] ainda não foi possível esclarecer todos os mecanismos que determinam o desenvolvimento do LPO.

Muitos estudos têm sido realizados na tentativa de esclarecer o comportamento biológico do LPO.[17,19,20] Neste contexto, sabe-se que a angiogênese desempenha importante papel no desenvolvimento e atividade de doenças inflamatórias crônicas,[50] bem como no processo de formação e progressão tumorais.[47,74] Nestas situações, percebe-se persistente ou excessiva ativação de agentes pró-angiogênicos,[26] dentre os quais os mais conhecidos são o VEGF, as angiopoietinas, FGF (fibroblast growth factor), TNF-α (tumor necrosis factor-alpha) e TGF-β (transforming growth factor-beta).[27]

Na presente pesquisa, a imunodeteção do VEGF, ANG-1 e ANG-2 foi avaliada em espécimes de LPOR e LPOAE, uma vez que estudos prévios mostraram diferenças na vascularização,[21,23,24] bem como na atividade proliferativa[75] entre estas formas clínicas, o que poderia refletir no comportamento mais agressivo do padrão erosivo da doença, sugerido por alguns autores.[76,77] Além disso, esses fatores de crescimento foram avaliados em espécimes de hiperplasia fibroepitelial, displasia epitelial e carcinoma espinocelular orais, buscando comparar o processo de neoformação vascular do LPO com o de lesões de diferentes comportamentos, a fim de encontrar uma relação entre esses fatores de crescimento angiogênicos e comportamento biológico.

Para a seleção da amostra do grupo LPO foi utilizada a combinação de critérios clínicos e histopatológicos, recomendada por Van der Meij e Van der Wall,[78] bem como as características preconizadas pela Organização Mundial da Saúde.[79] Embora Van der Meij e Van der Wall[78] sugiram que o diagnóstico de LPO deveria

ser considerado somente na presença do padrão reticular, associado ou não às outras formas clínicas da doença, no presente estudo os padrões reticular, em placa e papular foram incluídos no grupo LPOR e os padrões atrófico e erosivo no grupo LPOAE, desde que as características microscópicas preenchessem os critérios para a determinação do diagnóstico desta doença. Quando havia associação entre lesões brancas e atrófico/erosivas, as lesões eram incluídas no grupo LPOE, desde que as biópsias realizadas abrangessem essas áreas. Para a inclusão das lesões nos grupos-controle, consideraram-se as características microscópicas específicas de cada doença, porém para a inclusão de hiperplasias fibroepiteliais, preconizou-se a presença de infiltrado inflamatório e vascularização, evitando dessa forma, que a inclusão de lesões com características predominantemente fibrosas pudessem interferir nos resultados deste estudo.

Destaca-se que para comparar a imunodetecção dos agentes pró-angiogênicos entre as lesões de LPO e cada grupo-controle, optou-se por formar um único grupo LPO, pois não houve diferenças significativas entre os grupos LPOR e LPOAE quanto às ANG-1 e ANG-2. Além disso, a imunodetecção do VEGF foi inferior no grupo LPOAE, o que pode ter sido consequência do uso de corticosteróides tópicos para alívio dos sintomas provocados por estas lesões. O resultado dessas comparações revelou que a imunodetecção do VEGF no grupo LPO foi significativamente inferior à do grupo CEO e superior à do grupo HFO. Por outro lado, nenhuma diferença significativa foi observada em relação ao grupo DEO. Quanto à ANG-1, o grupo LPO apresentou imunodetecção significativamente superior em relação ao grupo HFO. A imunodetecção da ANG-2 não mostrou diferença significativa entre os grupos LPO e os controles, sendo encontrados valores muito semelhantes entre os grupos LPO e DEO. Assim, sugere-se que a

angiogênese no LPO possa se comportar de modo semelhante ao de lesões displásicas epiteliais.

Neste estudo, não foram encontradas correlações entre a ANG-1, ANG-2 e VEGF nos diferentes grupos estudados. Esse achado pode ter sido resultado do pequeno tamanho da amostra, mas também refletir a complexidade da angiogênese, a qual pode ser influenciada por uma série de outras citocinas pró-angiogênicas e tipos celulares para determinar a neoformação vascular.

A comparação do número de vasos sanguíneos entre os grupos LPOR e LPOAE não mostrou diferença significativa, contrariando estudos anteriores, nos quais o número de vasos foi superior no LPO atrófico/erosivo comparado com as lesões reticulares.[21,24] Portanto, não é possível confirmar que a angiogênese esteja relacionada com as diferentes formas clínicas desta doença, conforme sugerido por Tao et al..[21] Neste estudo, optou-se por não realizar a comparação do número de vasos entre o grupo LPO e os grupos-controle, por se tratarem de lesões de diferentes naturezas, dentre as quais umas apresentam um campo histológico com predomínio de células epiteliais, outras com predomínio de tecido conjuntivo, o que poderia interferir na fidelidade desta análise.

Ressalta-se que as imunodetecções do VEGF e ANG-1 foram significativamente superiores no grupo LPO comparado com o grupo HFO e que valores de imunodetecção da ANG-1, ANG-2 e VEGF foram semelhantes entre os grupos LPO e DEO, sugerindo que a angiogênese no LPO possa comportar-se de modo semelhante a lesões displásicas epiteliais. A falta de correlação entre estes fatores de crescimento nas lesões de LPO sugere o envolvimento de outras citocinas pró-angiogênicas, além daquelas avaliadas neste estudo, no processo de neoformação vascular desta patologia. Dessa forma, mais estudos são necessários

para a investigação de outras citocinas pró-angiogênicas, para que se possa compreender melhor o processo de angiogênese nesta doença.

6 CONCLUSÕES

De acordo com a metodologia e resultados observados neste estudo, pode-se concluir que:

- A imunodetecção do VEGF é significativamente superior no grupo LPOR comparado ao LPOAE.
- Não há diferença na imunodetecção da ANG-1 e ANG-2 e no número de vasos sanguíneos entre lesões de líquen plano oral reticular e atrófico-erosivo, portanto, a angiogênese pode não estar associada às diferentes formas clínicas da doença.
- Não há correlação entre os fatores de crescimento avaliados nas diferentes lesões estudadas, o que sugere o envolvimento de outras citocinas pró-angiogênicas, além daquelas avaliadas neste estudo, no processo de neoformação vascular e reflete a complexidade da angiogênese.
- A imunodetecção do VEGF e da ANG-1 é significativamente superior no grupo LPO em relação ao grupo HFO. Por outro lado, os percentuais de imunodetecção do VEGF, ANG-1 e ANG-2 são semelhantes entre os grupos LPO e DEO. Dessa forma, a angiogênese no LPO pode comportar-se de modo semelhante ao de lesões displásicas epiteliais.

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ANEXO A

Aprovação de Projeto pela Comissão Cientifica e de Ética da Faculdade de Odontologia da PUCRS

Comissão Científica e de Ética
Faculdade da Odontologia da PUCRS

Porto Alegre 04 de Julho de 2011

O Projeto de: Tese

Protocolado sob nº:	0044/11
Intitulado:	Imunodetecção do VEGF e das Angiopoietinas 1 e 2 em Espécimes de Líquen Plano Oral
Pesquisador Responsável:	Profa. Dra. Fernanda Gonçalves Salum
Pesquisadores Associados	Márcia Rodrigues Payeras
Nível:	Tese / Doutorado

Foi ***aprovado*** pela Comissão Científica e de Ética da Faculdade de Odontologia da PUCRS em *04 de Julho de 2011*

Este projeto deverá ser imediatamente encaminhado ao CEP/PUCRS

19 07 2011

Profa. Dra. Ana Maria Spohr
Presidente da Comissão Científica e de Ética da
Faculdade de Odontologia da PUCRS

Av. Ipiranga, 6681, Prédio 06 sala 210
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ANEXO B

Aprovação do Projeto pelo Comitê de Ética em Pesquisa da PUCRS

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

OF. CEP-1254/11

Porto Alegre, 12 de agosto de 2011.

Senhora Pesquisadora,

O Comitê de Ética em Pesquisa da PUCRS apreciou e aprovou seu protocolo de pesquisa registro CEP 11/05540 intitulado **"Imunodetecção do VEGF e das angiopoietinas 1 e 2 em espécimes de líquen plano oral"**.

Salientamos que seu estudo pode ser iniciado a partir desta data.

Os relatórios parciais e final deverão ser encaminhados a este CEP.

Atenciosamente,

Prof. Dr. Rodolfo Herberto Schneider
Coordenador do CEP-PUCRS

Ilma. Sra.
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APÊNDICE A
PDF do Artigo de Revisão publicado na Revista Archives of Oral Biology

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Review

Oral lichen planus: Focus on etiopathogenesis

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ABSTRACT

Lichen planus is a chronic mucocutaneous inflammatory disease, which frequently affects the oral mucosa of white females over 40 years old. Its aetiology remains uncertain and the pathogenesis is still the object of much speculation. The present paper presents the most well known antigens, and describes the action of different cells and proteins associated with the development of that disease, as well as the possible agents involved with its malignant transformation. Different external agents, especially virus, and internal agents, like stress, and the *heat shock protein* antigen expression, associated or not, can alter the basal keratinocytes of the oral mucosa making them susceptible to apoptosis by $CD8^+$ cytotoxic T cell as well as activate matrix metalloproteinase and mast cell degranulation, which produce a great range of inflammatory mediators and cytokines determining the clinical onset of the disease. Regarding carcinogenesis, since it is a complex process and presents multifactorial origin, it is believed that there may be a synergism between intrinsic, such as inflammation mediators, and extrinsic agents (tobacco, alcohol, viral infections) for the OLP malignant transformation to occur. However, further studies are needed to better understand the origin, pathogenesis and process of malignant transformation of OLP.

Contents

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1. Introduction

Lichen planus (LP) is a chronic mucocutaneous inflammatory disease that frequently affects the oral mucosa.[1,2] It tends towards white and middle-aged female patients.[3–5] Clinically, oral lichen planus (OLP) can present six different patterns: papule, reticular, plaque, atrophic, erosive and bullous[6] (Fig. 1), each showing specific characteristics and appearing in either isolated or associated forms.[1] Among those, the reticular is the most prevalent type characterised by the presence of Wickham striae, which are typically symmetric, bilateral, asymptomatic, and predominantly found in the buccal mucosa. The erosive form, although less frequent, presents greater clinical significance as the lesions are usually symptomatic, ranging from a slight discomfort to episodes of intense pain.[7] In the latter, the use of topical steroids are the first line to reduce symptoms and to improve the patients' quality of life. However, in case of persistent lesions systemic steroids are indicated.[8] Moreover, in situations of poor response of to the treatments above, alternative treatments ones can still be used,[8] such as calcineurin inhibitors,[9] antioxidants,[10] biologic therapies,[11] Photo Dynamic Therapy[12] and Laser Therapy.[13] OLP manifestations can persist for years alternating between periods of quiescence and exacerbation.[1]

Upon histopathological examination, OLP lesions present hyperkeratosis (in papular, plaque and reticular forms), hydropic degeneration of the basal cell layer of the epithelium and dense well-defined lymphocyte infiltrate, predominantly T, in the superficial subjacent connective tissue. The width of the spinous layer varies and the interpapillary ridges might not be present or be hyperplasic, but are often cuspidal or saw-tooth.[7,14–16]The follow up of OLP patients has shown some evidence of the disease malignant potential.[4,5,14,17–19] Despite some controversies on this issue, the OLP is actually a potentially malignant disorder.[20] Thus, a long-term monitoring of those patients is recommended, although the consultation frequency has not been established yet.[2,21]

OLP aetiology and pathogenesis has not been totally understood, however, some external and/or internal antigens have been suggested to trigger this disease, and different development mechanisms have also been hypothesised. Recently, Roospashree et al.[22] conducted a review discussing the various hypotheses on OLP pathogenesis. In the present review, besides the pathogenesis discussed through the performance of different cell types, chemokines and metalloproteinases, the aetiology of that disease and possible agents involved in the malignant transformation of OLP will be approached.

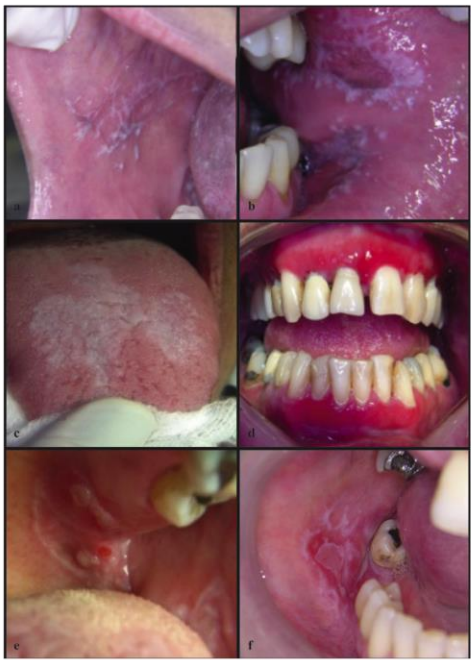

Fig. 1 – (a) Reticular OLP; (b) papule OLP; (c) plaque OLP; (d) atrophic OLP; (e) bullous OLP; and (f) erosive OLP.

2. Aetiology

The aetiology of the OLP remains uncertain, but some evidence points out that the disease is an immunological process triggered by an antigen that alters the basal keratinocytes of the oral mucosa making them susceptible to cell immune response. It induces the activation of $CD4^+$ T and $CD8^+$ T lymphocytes and cytokines production, such as interleukin-2 (IL-2), interferon gamma (IFN-γ) and tumour necrosis factor (TNF), which determine the keratinocytes apoptosis.[1,2,23–26]The antigen that triggers this inflammatory response is still unknown, it might have an intrinsical or an extrinsical origin,[2] besides, there are controversies whether one or two are involved.[15] In some cases, extrinsical antigens that include dental restoration[27–29] and drugs, specially antimalarials,[30] cardiovascular agents,[31–33] gold salts,[34] non-steroidal anti-inflammatory,[31,35] hypoglicemics[33] have been identified. In such cases, in spite of presenting lesions with clinical and microscopic features very similar to the OLP ones,[1,36,37] they are preferentially called oral lichenoid reaction (OLR).[21] Therefore, the diagnosis must be based on the recognition of clinical alterations as well as on carrying out an interview with the objective of observing a possible cause–effect relationship.[38,39]

Several reports have pointed out a possible association between OLP and viral infections. Four human herpes virus

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Table 1 – The prevalence of HPV in OLP cases published in the past 10 years (Pubmed).

Reference	N	Detection methods	Detection of HPV in specimens of LPO (%)
O'Flatharta et al.[45]	38	PCR	26.3% (16)
Ostwald et al.[46]	65	PCR	7.7% (6–11); 9.2% (16–18)
Campisi et al.[47]	71	PCR	19.7% (6, 16, 18, 31, 33)
Giovanelli et al.[48]	49	PCR	24.50% (6, 16, 18, 33, 53)
Cianfriglia et al.[49]	15	ISH	20% (6–11, 31–33–51, 16–18)
Khovidhunkit et al.[50]	16	PCR	0% (NA)
Szarka et al.[51]	119	RT-PCR	32.8% (6, 11, 16, 18, 31, 33)
Razavi et al.[52]	29	PCR	31% (18)
Yildirim et al.[40]	65	IHC	21% (16)
Mattila et al.[53]	82	PCR	15.9% (6, 11, 16, 31, 33)

ISH, in situ hybridisation; PCR, polymerase chain reaction; RT-PCR, real time polymerase chain reaction; IHC, immunohistochemistry; in parenthesis, HPV probe positive; NA, not available.

families subtypes have been associated with oral manifestations of lichen planus: herpes simplex,[40] Epstein-Barr,[40,41] Cytomegalovirus[42,43] and Herpes virus 6,[43,44] However, some doubts remain whether these agents are associated with the OLP or whether the infection superimposes the lesions already in existence.[25]

The most extensively investigated viruses in the OLP aetiology are the human papillomavirus (HPV) and the hepatitis-C virus (HCV). Present investigations have shown that, most of the time, a high identification of HPV in OLP lesion (Table 1) and recent systematic review shows the strong association between HPV and OLP.[54] Furthermore, it has been proved that the HPV prevalence increased gradually with increasing severity of the lesions.[46,51,55] These findings suggest that HPV may play some etiological role in OLP, but also be associated with the malignant progression of potentially malignant oral disorders. However, it is believed that frequent ulcerative lesions in OLP that make it more susceptible to HPV infection,[54] or the chronic use of steroids could enhance replication of HPV virus.[54,55] Therefore, it is impossible to believe that the isolated presence of this virus is associated with the OLP aetiology, as well as, with its malignant transformation, but that its presence could enhance the effect of carcinogens agents, increasing the malignant transformation risk.[51,56]

A positive association between HCV and OLP has been recorded, especially in the populations from the Mediterranean countries, the United States, Saudi Arabia, Taiwan and Nigeria, when compared with other regions in the world (Table 2), suggesting a possible geographic heterogeneity.[74] However, three recent meta-analyses have demonstrated strong evidence between HCV and OLP in all regions of the world.[91–93] This association could be explained by the ability of the HCV virus to infect other cells in addition to hepatocytes, as epidermal cells and the high mutation rate of the virus results in repeated activation of the immune cells, increasing the risk of developing autoimmune diseases.[94] Besides, it is known that in patients with chronic liver disease, the treatment with interferon gamma (INF-γ) may lead to the oral lichenoid reaction to that drug.[95] Furthermore, HCV-associated OLP appears to be a distinct subtype of that disease, since studies have shown an increased frequency of HLA (Human Leucocyte Antigens) class II allele, HLA-DR6 in these cases compared with OLP patients without hepatitis C virus.[95–98] Thus, further studies need to be conducted in order to clarify the role played by the HCV infection in the OLP pathogenesis.

Regarding the association between OLP and viral infections it can still be pointed out that some cases of this disease have been associated with HIV infection. Nevertheless, the lesions might be more related to antiretroviral therapy than to actually the infectious agent.[99,100]

With respect to the antigen of intrinsic origin, the *heat shock protein* (HSP) stands out, expressed by all cell types,[101] functioning essentially for cell communication, differentiation and growth, signal transduction and apoptosis.[102] The increase in this protein expression can occur in response to several exogenous agents, such as temperature change, medications, viruses, nutrients deprivation and growth factors. This protein high expression in OLP suggests that it might be an auto-antigen of the disease.[103] The identification of an auto-antigen in the OLP lesions associated with the characteristics of the disease, such as chronicity, association with other autoimmune illnesses, preference for adult female patients, presence of lymphocyte T in the lesions, and effectiveness of the immunosuppressive therapy reinforces the autoimmune theory.[104] However, as described above, the HSP super-expression can occur due to several exogenous agents and, therefore, be a retarded event, not the disease trigger.[103,105] In spite of the weak expression of TGF-β1 – protein with immunosuppressive effects – failure of T cells apoptosis induced by keratinocyte and Langerhans cell maturation, and keratinocyte apoptosis in OLP are other situations that suggest the autoimmune origin for this disease.[22]

Psychological disorders, such as depression, anxiety and stress have been investigated in the OLP aetiology, as patients with the disease report more frequent development or exacerbation of lesions during periods of greater emotional tension.[106–110] However, studies that have evaluated those disorders in OLP patients have shown controversial results. Some authors found a positive association between OLP and presence of psychological alterations,[108,111–113] while others did not find that association.[107,114,115] The use of different inventories, as well as their subjectivity and the lack of methodological standardisation of studies are responsible for the controversial results. Moreover, OLP etiopahogenesis is complex and presumably dependent on the interaction of different factors. It is believed that stressful situations could influence the development of the disease, modifying and promoting dysregulation of immune functions with alteration

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Table 2 – The prevalence of HCV in OLP cases published from January 2001 to December 2012 (Pubmed).

Reference	N	Country	Detection of HCV in specimens of LPO (%)
Beaird et al.[57]	24	USA	16.6
Erkek et al.[58]	54	Turkey	12.9
Kirtak et al.[59]	73	Turkey	6.8
Daramola et al.[60]	57	Nigeria	15.7
Eisen[14]	195	USA	0
Figueiredo et al.[61]	68	Brazil	8.8
Garg et al.[62]	86	Nepal	0
Mignogna et al.[63]	600	Italy	27.5
Prabhu et al.[64]	65	India	0
Gimenez-García and Pérez-Castrillón[65]	101	Spain	8.9
Klanrit et al.[66]	60	Thailand	6.6
Mahboob et al.[67]	184	Pakistan	23.3
Bokor-Bratic[68]	48	Serbia and Montenegro	0
Campisi et al.[69]	859	Italy	27.7
Chung et al.[70]	32	Taiwan	43.7
Denli et al.[71]	140	Turkey	5
de Mattos Camargo et al.[72]	50	Brazil	2
Ghodsi et al.[73]	146	Iran	4.7
Harman et al.[74]	128	Turkey	6.2
Karavelioglu et al.[75]	41	Turkey	4.8
Lodi et al.[76]	303	Italy	19.1
Assad and Samdani[77]	114	Saudi Arabia	26.3
Luis-Montoya et al.[78]	36	Mexico	2.7
Rahnama et al.[79]	66	Iran	1.5
Das et al.[80]	104	India	1.9
Khaja et al.[81]	52	India	44
Ali and Suresh[82]	40	Saudi Arabia	0
Giuliani et al.[83]	82	Italy	11.4
Yarom et al.[84]	62	Israel	4.8
Stojanovic et al.[85]	173	Slovenia	1.2
Lin et al.[86]	104	Taiwan	22.1
Zhou et al.[87]	232	China	1.7
Konidena and Pavani[88]	25	India	12
Nagao and Sata[89]	59	Japan	67.8
Jayavelu and Sambandan[90]	30	India	0

of the balance of Th1/Th2 cytokines and increased Th2 response.[116–119]The genetic predisposition has been hypothesised in OLP aetiology.[120] In this context, many studies have focused on the relationship between HLA and OLP, demonstrating that the HLA-DR1 is frequently associated with cutaneous idiophatic LP, but not in OLP[121] and the HLA-DR6 is usually linked to hepatitis C virus-associated OLP.[95–97] Moreover, Th1/Th2 cytokine polymorphisms have been investigated. Between them IL-18 and IL-4,[122,123] IL-6,[124] TNF-α,[123,125,126] IL-10[126,127] and INF-gama[123,125] seem to have some influence on the susceptibility and progression of OLP. Recently, one mutation in the chromosome 3p14-3q13 has been identified in genetic linkage analysis study, which was suggested as a possible responsible factor for OLP in a Chinese family with five affected individuals.[128] However, it is still too early to say that the disease is genetically determined as there is the need of confirmation by further studies in different geographical areas.[2]

3. Pathogenesis

Several cell types, proteins of the extra-cellular matrix and chemokines, contribute to the onset of the OLP through the activation of different pathways. The presence of cells that involve migration and activation of T cells and killing of keratinocytes produce antigen-specific cell-mediated immune response, however, performance of matrix metalloproteinases, chemokines and mast cell are responsible for non-specific immune response.[15,22] Finally, circulating autoantibodies to desmoglein 1 and 3[129] and identification of IgA and IgM,[130] suggest a role of humoral immunity in OLP.

4. Cells involved in the pathogenesis of oral lichen planus

The cells involved in the OLP pathogenesis are: keratinocytes, $CD8^+$ T lymphocytes (*Suppressor*), $CD4^+$ T lymphocytes (*Helper*), dendritic cells, mastocyte and macrophages whose levels vary according to the stage in which the lesions are found. In OLP early stages, the helper-T lymphocyte levels, the macrophages and the dendritic cells are higher than the rates presented by more advanced stage lesions, which present high levels of supressor-T lymphocytes.[131] That distribution suggests that in the early stages there is the predominance of antigen-presenting cells, as well as those cells responsible for inducing an inflammatory response, and the predominance of defence cells occurs in more advanced stages, which will determine the keratinocyte apoptosis.[132]

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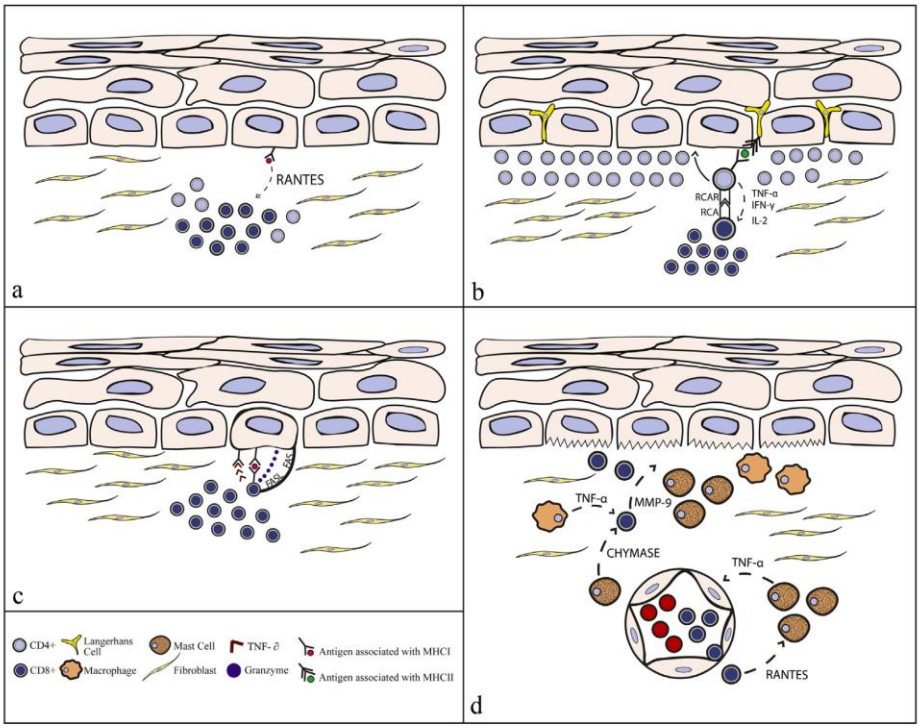

Fig. 2 – (a) Keratinocytes activated by antigens associated with class I MHC secrete chemokines, which attract lymphocytes; (b) T CD4+ lymphocytes activated by antigens associated with class II MHC expressed by LCs activate the cytotoxic T lymphocytes (c) Apoptosis of keratinocytes via different mechanisms; (d) interaction between mastocytes and T lymphocytes which, through a cyclic mechanism, might promote OLP chronicity. Chymases inducing MMP-9 production by the T lymphocyte, which proves the indirect action that the mastocytes can have in the destruction of the basement membrane.

4.1. *Keratinocyte*

Keratinocyte, the cell that forms the epithelium of the oral mucosa, is associated with the secretion of type IV collagen and laminin V, proteins that form the basement membrane,[133] which, in turn, is essential for maintaining the keratinocyte vitality. This shows a close relationship and dependence between both structures.[15] In the OLP, the keratinocyte is the target cell, the one to suffer apoptosis,[1,15,104] however, for this to occur this cell needs to express an antigen – still unknown – in the early stages of the disease development.[15] Once active, those keratinocytes secrete chemokines, which attract lymphocytes and other immune cells that can induce OLP development, as well as favour the disease chronicity[134,135] (Fig. 2a).

4.2. *Dendritic cell (DCs)*

DCs present an important role in the immunological response, as they activate T cells through antigenic stimulation.[136] Studies have revealed an increase in the number of DCs in OLP, indicating that they may be associated with its pathogenesis.[137–140] According to Santoro et al.,[138] the increase of different subsets of DCs, such as Langerhans Cells (LCs), stromal DCs and plasmacytoid dendritic cells (PDCs) may promote inflammatory response in OLP. Among these, LCs are the most dendritic cell studied. Those cells reside in the supra-basal layers of the stratified epithelium of the skin and oral mucosa whose function is to capture and antigens. When these cells capture the antigens, they are activated, migrate to the regional lymphonodes and are introduced to the T lymphocytes, producing a primary immune response. Thus, when the LCs recapture that antigen, it will be recognised by the T lymphocytes circulating memory, which will induce a secondary immune response.[141,142] In the OLP lesions, a high number of LCs are present on the basal layer of the epithelium.[24] It is suspected that in this lesion the LCs play an important role in presenting antigens to the T lymphocyte through class II Major Histocompatibility Complex (MHC) molecules,[143] introducing not only an initial sensitivity to the antigen (primary immune response) but also a subsequent secondary immune response which permits the appearance of the disease clinical signs.[141]

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4.3. *CD4⁺ T cell*

CD4⁺ T lymphocytes are cells capable of activating the B lymphocytes, macrophages and T CD8⁺ lymphocyte and thus, responsible for orchestrating different cells during the immune response. These cells are classified into three sub-groups: Th1, Th2 and Th17, morphologically undistinguishable but distinct by cytokine production. Th1 sub-group is characterised by the production of IFN-γ, IL-2 and TNF-α, which are macrophage and cytotoxic T lymphocytes activating cytokines. Th2 sub-group secretes IL-4, IL-5, IL-10 and IL-13, elements that are crucial for the production of antibodies. Th17 sub-group, recently discovered, produces IL-26, IL-22 and IL-17. The latter, an important inducer to inflammatory response, when uncontrolled, is associated with different autoimmune conditions, such as multiple sclerosis, psoriasis and lupus.[144] Recently, the proportion of Th1 and Th17 cells and serum IL-17 levels in patients with OLP were significantly greater than controls, especially in the atrophic-erosive OLP group compared with reticular OLP group, suggesting that Th17 cells and their cytokine Th17 might play an important role in OLP pathogenesis.[145] In the OLP lesions, the CD4⁺ T cells form most of the lymphocytes present in small clusters deeper down in the subepithelial lymphocyte-rich band[146,147] or in the lamina propria[23]; however, they are not present in the areas of basement membrane rupture.[148] In this disease, the CD4⁺ T lymphocytes can be activated, at least partially, by antigens associated with class II MHC expressed by LCs or by the keratinocytes. From this point, the CD4⁺ T lymphocytes activate the cytotoxic T lymphocytes by linking *Request to Cytotoxic Activity* (RCA) receptor with RCA on the surface of CD8⁺ T lymphocyte or by secreting Th1 derivated cytokines, such as IFN-γ, IL-2 e TNF-α[15] (Fig. 2b). In addition, the IFN-γ local production can maintain the class II MHC expression through keratinocytes, thus contributing to the lesion chronicity.[149]

4.4. *CD8⁺ T cell*

CD8⁺ T lymphocytes are cells that recognise antigens presented by the class I MHC molecules determining its elimination by cytotoxity.[144] In the OLP, the T CD8⁺ cell infiltrate is mainly found in the intra-epithelial region and in the basement membrane disruption areas, adjacent to the basal keratinocytes destruction.[24,148,150] It should be pointed out that the antigen identification, expressed by the basal keratinocytes can occur either through its routine vigilance or attraction, promoted by the chemokines produced by activated keratinocytes.[15] At the end of this process, the caspase cascade occurs via different mechanisms: (1) activation of perforins and granzymes; (2) expression of Fas L receptor (CD95) that interacts with the Fas molecule on the surface of the keratinocytes, and (3) TNF-α secretion, which is linked to TNF-α receptor in the target cell, determining the keratinocyte apoptosis[15] (Fig. 2c).

4.5. *Mastocyte cell (MCs)*

Mastocyte cells are derived from the CD34⁺ haematopoietic progenitor that have the ability to activate T lymphocyte, suffer degranulation, and release a series of mediators that modulate the inflammatory response.[142] There is an increase in the MCs density in the OLP lesions, which are preferentially located in the lamina propria, near blood vessels and nerves.[151,152] Moreover, MCs density in this disease is markedly higher in the basement membrane rupture sites, suggesting that this cell might play a direct role in the basement membrane destruction, as well as in the T CD8⁺ lymphocyte migration to the intra-epithelial region.[148] However, recently, a greater number of mast cells has been identified in deeper connective tissue.[153] Approximately 60% of these mastocytes are found degranulated, releasing cytokines such as IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13 and IL-16, TNF-α, Chymase and RANTES.[15,154,155] It is known that IL-16 stimulates T lymphocyte direct migration through the connective tissue and that the TNF-α can regulate the secretion of adhesion molecule and favour the release of lymphocytes to the extra-vascular region. These lymphocytes, in turn, secrete RANTES and other mediators, stimulating the mastocytes to release TNF-α. This proves there is an interaction between mastocytes and T lymphocytes, which, through a cyclic mechanism, might promote OLP chronicity.[22,131] Chymases are proteases capable of inducing MMP-9 production by the T lymphocyte,[156] which proves the indirect action that the mastocytes can have in the destruction of the basement membrane[157] (Fig. 2d).

4.6. *Macrophages*

Macrophages are phagocyte cells derived from blood monocytes, recruited for the tissues in the presence of chemotaxis signals. They are present in the healthy oral mucosa and in larger numbers during pathological processes.[158] Macrophages are classified in M1 (pro-inflammatory) and M2 (anti-inflammatory) according to the functions of their effectors.[159] The M1 macrophages might exacerbate OLP manifestation through the production of pro-inflammatory agents such as TNF-α e IL-1β. Those agents, in turn, regulate the presence of adhesion molecules on the endothelial cells surface inducing the production of chemokines (RANTES) by the keratinocytes, resulting in an increase in the inflammatory cells recruitment inside the lesion. Furthermore, it must be pointed out that the production of TNF-α by the macrophages can initiate the basal keratinocyte apoptosis and, indirectly, increase the disruption rate of the basement membrane by MMP-9, produced by T cells[158] (Fig. 2d).

5. Chemokines involved in the pathogenesis of oral lichen planus

Chemokines form a family of little cytokines, initially identified by their modulator action on the inflammatory response. More attention has been given to these proteins nowadays, especially due to their function on the endothelial cells.[160] Besides, recent evidence has shown the role chemokines play on different autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis.[161] One of the most widely studied chemokines is Regulated on Activation Normal T-cell Expressed and Secreted (RANTES), a member of

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the CC chemokine family, produced by different cell types, including activated T lymphocyte, keratinocytes and mastocytes. Its biological effect, especially the recruitment of lymphocyte, natural Killer cells and mastocytes, is produced when RANTES is linked to different receptors, such as CCR-1, CCR-3, CCR-4, CCR-5, CCR-9 and CCR-10, found on the cell surface.[15] It has been proved, in vitro, that RANTES production by the T cells initiates mastocyte degranulation, which liberates TNF-α and chymase and, in turn, stimulates the release of RANTES by the cells, attracting more mastocytes and their consequent degranulation. Therefore, this cyclical mechanism can contribute to the OLP lesions chronicity.[15,25,155]

6. Matrix metalloproteinases (MMPs) involved in the pathogenesis of oral lichen planus

The MMPs form a family of zinc-dependent proteases, with at least 20 members involved in cell migration, angiogenesis and proteolytic activation of growth factors, events necessary for the normal tissue repair as well as in wound healing and tumoural invasion.[162] Their function is in part regulated by tissue inhibitor of metalloproteinases (TIMPs), among which, the most well known are TIMP-1, TIMP-2 and TIMP-3. An imbalance between MMPs and TIMPs can be associated with the tissue destruction seen in some pathologies, like cancer, arthritis and cardio-vascular diseases.[163,164] The MMPs that come from T-cells take part in the movement of those cells in extravascular tissues and in their migration through the basement membrane.[15] Studies have identified a difference in the MMP-2 expression and distribution in the OLP. Sutinen et al.[165] verified that the MMP-1 expression, besides being low, was restricted to fibroblasts of the sub-epithelial region, while MMP-2 was not detected in the 10 samples studied. Zhou et al.[157] found that MMP-2 and MMP-3 were mainly expressed in the epithelial region, and MMP-9 was identified in the sub-epithelial inflammatory infiltrate. Still, according to those authors, MMP-9 induces the rupture of collagen IV, and thus might be associated with the basement membrane degradation and facilitate the T-cell intra-epithelial degradation. In a Rubaci et al.[166] study the expression of MMP-2 and MMP-7 in epithelium and connective tissues from OLP lesions were greater than normal oral mucosa. Likewise, the MMP-2/TIMP-1 and MMP-7/TIMP-1 ratios were higher in the OLP patient group than in the control group. These results suggest that increased MMPs expression and imbalance between MMPs and TIMPs play a role in the pathology of OLP. It has been pointed out that different OLP clinical forms (erosive and non-erosive) are associated with significant differences in the MMPs expression levels, especially MMP-1, MMP-2, MMP-3 and MMP-4, which seem to be more associated with the development of erosive lesions.[167,168]

7. Role of immune cells and their cytokines in malignant transformation of OLP

Although the causes for the malignant transformation in OLP patients have not been totally clarified, it is believed that nutritional disorders, genetic factors and immunosuppression induced by certain therapies used in OLP treatment, as well as the action of some external mutagenic agents (tobacco, alcohol, candidiasis and HPV virus) could make the oral mucosa more sensitive, increasing malignancy risk.[15,120]

Besides, the evidence has shown a close relationship between chronic inflammation and tumoural growth and development.[169,170] Several chemical mediators are released during the chronic inflammatory process that in the long term can influence the expression of genes that control proliferation and apoptosis, thus promoting carcinogenesis.[158,169,171]

Among the main pro-inflammatory cytokines involved in this process, those derived from TAM (tumour-associated macrophages) and Th17, such as TNF, IL-1β, IL-6, IL-12, IL-23, stand out. They can activate transcription factors, like AP-1 (Activator Protein), NFKB (Nuclear Factor KB) and STAT-3 (Signal Transducer and Activator of Transcription 3), which promote the expression of many other mediators with pro-inflammatory, pro-angiogenic and immunoregulatory activities thus performing an important role in this disease malignisation.[158,169,171] However, the role of some cytokines can vary according to the intensity of their expression[15] and tumour development stage.[163] The TGF-β can inhibit tumour growth in early carcinogenesis stages but, on the other hand, it can favour neoplasia growth especially for inducing angiogenesis and MMP-9 expression in more advanced stages.[172] Other inflammation mediators like chemokines and their receptors have been investigated in tumour initiation and promotion phases.[173] Molecular studies have shown that RANTES can induce the expression of important cell enzymes such as phosphatidylinositol (PI) 3-Kinase and Akt/protein kinase B, which can induce pro-proliferation signals that influence cell survival and malignant transformation.[174,175]

In addition, an imbalance between MMPs and TIMPs can be associated with the OLP malignant transformation, being MMP-2 and MMP-9 a possible marker of the malignant transformation potential of the disease.[166,168]

Chronic inflammation can also induce cyclooxygenase (COX) release, an enzyme that transforms arachidonic acid into prostaglandin; its COX-2 isoform, when super-expressed, has been associated with important carcinogenesis stages[176] such as angiogenesis[177] and apoptosis.[178] A COX-2 super-expression has been identified in the OLP, so it was suggested that it could be associated with the increase of its malignant transformation potential.[179]

More recently, oxidative stress increase and imbalance in the antioxidant defense system have been found in the OLP patients oral fluids.[180–182] Battino et al.[180] have found low levels of saliva uric acid and an increase in serum gamma glutamyl transpherase (GGT) and total antioxidant capacity of saliva in patients with OLP compared with control patients. Similarly, Ergun et al.[181] showed that, total antioxidant defense (TAA) serum in OLP patients was significantly lower than that in healthy subjects and that salivary lipid peroxidation product malondialdehyde (MDA) levels were significantly higher in the OLP group compared with control group. Results of Kawanishi et al.[182] revealed that nitrative and oxidative DNA lesion products were expressed in epithelial cells and inflammatory cells at the carcinogenesis site in human and animal models. Thus, it

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suggests that those elements contribute to the development of DNA damage and malignant transformation of inflammation-associated carcionogenesis.

Finally, cytokines from T $CD4^+$ lymphocytes, like IFN-γ, TNF-α and IL-12, and the cytotoxic activity of lymphocyte $CD8^+$ also present in the chronic inflammatory response play an important role in the inhibition and death of malignant cells. Thus, the appearance of malignant phenotypes in OLP patients can be associated with an imbalance between the activity of different kinds of cells and the expression of different inflammation mediators, inhibitors and carcinogenesis promoters.

8. Conclusion

Despite the extensive literature regarding the OLP origin and development mechanism, its aetiology remains uncertain and the pathogenesis is still the object of much speculation. One believes that different external agents, especially virus, and internal agents, like stress, and the HSP antigen expression, associated or not, can trigger OLP. Subsequently, lymphocytes, the main cell to form OLP lesions, produce and respond to a great range of inflammatory mediators and cytokines that can affect the keratinocytes and stimulate their apoptosis, determining the clinical onset of the disease.

It remains uncertain, in spite of much discussion, whether OLP has the potential for intrinsic malignant transformation, or if external factors are associated with its malignisation process. Since carcinogenesis is a complex process and presents multifactorial origin, it is believed that there may be a synergism between intrinsic (inflammation mediators) and extrinsic agents (tobacco, alcohol, viral infections) for the OLP malignant transformation to occur.

Besides, it is believed that Th17, recently discovered, should be further investigated in OLP lesions, once it has been identified in different autoimmune diseases and is associated with protein production that can be involved in the malignisation of chronic inflammatory processes. Furthermore, studies aiming at elucidating the role of oxidative stress and other cytokines, especially, TGF-β, chemokines and MMPs in the OLP malignant transformation must be carried out, as they perform important functions associated with chronicity and disease aggressiveness.

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Competing interests

None declared.

Ethical approval

None.

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APÊNDICE B

Normas para a submissão de artigos científicos no periódico International Journal of Oral Maxillofacial Surgery

Guide for Authors

Would authors please note that the reference style for the journal has now changed. Please pay special attention to the guidelines under the heading "References" below

Authors wishing to submit their work to the journal are urged to read this detailed guide for authors and comply with all the requirements, particularly those relating to manuscript length and format. This will speed up the reviewing process and reduce the time taken to publish a paper following acceptance.

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Upon submission you will be required to complete and upload this form (pdf version or word version) to declare funding, conflict of interest and to indicate whether ethical approval was sought. This information must also be inserted into your manuscript under the acknowledgements section with the headings below. If you have no declaration to make please insert the following statements into your manuscript:
Funding: None
Competing interests: None declared
Ethical approval: Not required
Patient permission: Not required

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Authorship
All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data
(2) drafting the article or revising it critically for important intellectual content
(3) final approval of the version to be submitted.

Normally one or two, and no more than three, authors should appear on a short communication, technical note or interesting case/lesson learnt. Full length articles may contain as many authors as appropriate. Minor contributors and non-contributory clinicians who have allowed their patients to be used in the paper should be acknowledged at the end of the text and before the references.

The corresponding author is responsible for ensuring that all authors are aware of their obligations.

Before a paper is accepted all the authors of the paper must sign the Confirmation of Authorship form. This form confirms that all the named authors agree to publication if the paper is accepted and that each has had significant input into the paper. Please download the form and send it to the Editorial Office. (pdf version or word version) It is advisable that to prevent delay this form is submitted early in the editorial process.

Acknowledgements
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Work on human beings that is submitted to the International Journal of Oral and Maxillofacial Surgery should comply with the principles laid down in the Declaration of Helsinki (Recommendations guiding physicians in biomedical research involving human subjects. Adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964, amended by the 29th World Medical Assembly, Tokyo, Japan, October 1975, the 35th World Medical Assembly, Venice, Italy, October 1983, and the 41st World Medical Assembly, Hong Kong, September 1989). The manuscript should contain a statement that the work has been approved by the appropriate ethical committees related to the institution(s) in which it was performed and that subjects gave informed consent to the work. Studies involving experiments with animals must state that their care was in accordance with institution guidelines. Patients' and volunteers' names, initials, and hospital numbers should not be used.

Patient confidentiality

Patients have a right to privacy. Therefore identifying information, including patients' images, names, initials, or hospital numbers, should not be included in videos, recordings, written descriptions, photographs, and pedigrees unless the information is essential for scientific purposes and you have obtained written informed consent for publication in print and electronic form from the patient (or parent, guardian or next of kin where applicable). If such consent is made subject to any conditions, The Editor and Publisher must be made aware of all such conditions. Written consents must be provided to the Editorial Office on request. Even where consent has been given, identifying details should be omitted if they are not essential. If identifying characteristics are altered to protect anonymity, such as in genetic pedigrees, authors should provide assurance that alterations do not distort scientific meaning and editors should so note. *If consent for publication has not been obtained, personal details of patients included in any part of the paper and in any supplementary materials (including all illustrations and videos) must be removed before submission.*

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The following contributions will be accepted for publication. *Please take careful note of the maximum length where applicable*. Overlength articles will be returned to the authors without peer review:
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• clinical papers: no more than 5000 words and 30 references
• research papers: no more than 6000 words and 40 references
• review papers - no limit on length or number of references
• technical notes (surgical techniques, new instruments, technical innovations) - no more than 2000 words, 10 references and 4 figures
• case reports - no more than 2000 words, 10 references and 2 figures
• book reviews
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• IAOMS announcements
• general announcements.

Please note: Case reports will be considered for publication only if they add new information to the existing body of knowledge or present new points of view on known diseases.

All authors must have contributed to the paper, not necessarily the patient treatment. Technical notes and case reports are limited to a maximum of 4 authors, in exceptional circumstances, 5.

Criteria for Publication
Papers that will be considered for publication should be: • focused
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• well written in simple, scientific English grammar and style
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General points
Papers should be submitted in journal style. Failure to do so will result in the paper being immediately returned to the author and may lead to significant delays in publication. Spelling may follow British or American usage, but not a mixture of the two. Papers should be double-spaced with a margin of at least 3 cm all round.

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Papers should be set out as follows, with each section beginning on a separate page: • title page
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• text
• acknowledgements
• references

• tables
• captions to illustrations.
Please note that the qualifications of the authors will not be included in the published paper and should not be listed anywhere on the manuscript.

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200 words maximum. Do not use subheadings or abbreviations; write as a continuous paragraph. Must contain all relevant information, including results and conclusion.

Text
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• Present first the nature and scope of the problem investigated
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• Discuss - do not recapitulate results • Point out exceptions and lack of correlations. Do not try to cover up or 'fudge' data • Show how results agree/contrast with previous work • Discuss the implications of your findings • State your conclusions very clearly

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APÊNDICE C

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