Concomitant upregulation of matrix metalloproteinase-2 in lesions and circulating plasma of oral lichen planus

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Introduction

Oral lichen planus (OLP) is a chronic inflammatory mucosal disease of unknown origin which affects approximately 4% of the adult population, and more frequently affects middle-aged and elderly women.1,2 Lesions are usually bilateral and mainly affect the buccal mucosa, gingiva, and lateral side of the tongue.3 Clinically, OLP has a distinct morphology with two typical forms: atrophic-erosive lesions with or without concomitant reticular lesions, and reticular and/or plaque lesions.4 A diagnosis of OLP is based on the presence of an epithelial-connective tissue interface band of infiltrating T lymphocytes, and the lesion is considered to be an autoimmune disorder mediated by T cells.5

Background/purpose: Oral lichen planus (OLP) is a chronic inflammatory disorder characterized by a T cell-mediated immune response against epithelial cells. Matrix metalloproteinases (MMPs) are an important group of zinc enzymes and are thought to play an important role in the degradation of components of the extracellular matrix. The aim of this study was to assess the expression of MMP-2 and MMP-9 in both tissue specimens and circulating plasma.

Materials and methods: Twelve cases of OLP were collected. In addition, six cases with chronic inflammation and six normal subjects were also recruited. Oral tissue specimens were collected for measurement of MMPs by immunohistochemistry, and sera prepared from peripheral blood were used for gelatin zymography to determine MMP levels in plasma.

Results: The level of MMP-2 in patients with OLP was significantly higher than that in the other two groups, both in tissues and sera (P<0.0001). However, there was no difference in the expression of MMP-9 among these groups.

Conclusion: MMP-2 overexpression in OLP is consistent with its upregulation in peripheral serum. This result also indicates that MMP-2 might play a role in the pathogenesis of OLP.
Usually, OLP has a chronic progression, and most cases exhibit inflammation. The levels of some molecules involved in mediating inflammatory processes were reported to be increased in this disease. Among the effects of the inflammatory mediators, the induction of matrix metalloproteinases (MMPs) has been demonstrated. MMPs are a large family of proteolytic enzymes, which are involved in degrading many different components of the extracellular matrix. They are associated with normal tissue remodeling, embryonic development, wound healing, angiogenesis, bone resorption, and inflammation.

Histopathologically, a dense subepithelial band-like infiltration of lymphocytes and increased numbers of intraepithelial lymphocytes are found in OLP, with changes and disruption of the epithelial basement membrane. Basement membrane damage, basal cell liquefaction, and tissue remodeling associated with OLP require degradation of the surrounding extracellular matrix. In the MMP family, gelatinase (MMP-2 and MMP-9) cleaves denatured collagens (gelatins) and type IV collagen, the major component of basement membranes. Previous studies using immunohistochemistry showed that MMP-2 and MMP-3 were mainly found in OLP epithelium, while MMP-9 staining was found in the inflammatory infiltrate.

To the best of our knowledge, no one has elucidated the circulating plasma expression of MMPs in OLP. There is no available information on the interrelationships of MMP-2 and MMP-9 expression in oral lesional sites and circulating plasma. Therefore, our aim was to explore the expression of MMP-2 and MMP-9 in both oral tissue specimens and sera from peripheral blood of OLP patients using immunohistochemistry and gelatin zymography.

Materials and methods

Patient selection

Formalin-fixed, paraffin-embedded OLP specimens were obtained from 12 consecutive patients (9 females and 3 males) at the Department of Oral and Maxillofacial Surgery, Buddhist Dalin Tzu Chi General Hospital, Chiayi, Taiwan. Patients with a history of any systemic disease were excluded from the study. Their ages ranged from 28 to 83 years (mean, 61.08±15.6 years). The diagnosis of each lesion was confirmed by a histologic examination of hematoxylin and eosin-stained sections. The pathologic diagnosis of OLP was established as described by Krutchkoff et al. The types of OLP were subclassified into two clinical forms: reticular and/or plaque lesions (6 cases) and erosive lesions (6 cases).

Normal controls (clinically normal oral mucosa) were obtained from six healthy volunteers (mean age, 32.66±10.2 years; range, 21–58 years). Disease controls consisted of six specimens of oral chronic ulcer (ulceration persisting for more than 1 month) (mean age, 66.66±4.3 years; range, 52–70 years). In addition, peripheral blood from eight OLP patients (four cases from each subgroup), four cases of chronic ulcer, and four cases of normal oral mucosa were collected at the same time. Written consent was obtained from all patients before taking specimens. The study protocol was approved by the Research Ethics Committee of our institution.

Immunohistochemistry

Immunohistochemical staining was performed using a peroxidase-labeled streptavidin-biotin technique. Briefly, sections of 5µm were deparaffinized and then heated in an autoclave for 40 minutes to retrieve the antigenicity before blocking with endogenous peroxidase. Following treatment with 3% hydrogen peroxide in methanol for 10 minutes to quench endogenous peroxidase activity, sections were incubated with goat polyclonal immunoglobulin G (IgG) anti-MMP-2 (sc-8835; 1:20 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-MMP-9 antibodies (sc-6840; 1:20 dilution; Santa Cruz Biotechnology), or leukocyte common antigen, a specific antibody for lymphocytes (Dako, Carpinteria, CA, USA; 1:50 dilution), for 30 minutes. After washing in 10mmol/L Tris-buffered saline, sections were treated with donkey anti-goat IgG-HRP (sc-2020; 1:100 dilution; Santa Cruz Biotechnology) for 30 minutes. Final products were visualized by a diaminobenzidine (DAB) substrate kit (Liquid DAB Substrate Chromogen System, code K3467; Dako) for 5 minutes. Sections were then counterstained with hematoxylin, mounted with mounting medium, and examined by light microscopy. To demonstrate the specificity of staining, negative controls were included, in which the primary antibody was replaced with phosphate-buffered saline. A reddish-brown substrate in the cytoplasm indicated positive staining for MMP-2/MMP-9.

Each specimen was graded at 200× magnification as follows: +++, 50% to approximately 100%; ++, 25% to approximately 50%; and +, <25% of the proportion of positively stained cells in tissue sections.

Gelatin zymography

Serum samples were analyzed for MMP-2/MMP-9 with gelatin zymography as previously described. Peripheral blood was first collected then centrifuged at 400g for 10 minutes at room temperature. The plasma and buffy coat layers were then separated.

Peripheral blood was first collected then centrifuged at 400g for 10 minutes at room temperature. The plasma and buffy coat layers were then separated.
An additional centrifugation at 1000g for 10 minutes was performed on the plasma fraction to remove remaining cellular debris and the plasma was then stored at −80ºC. For each serum sample, 10μg of total protein was loaded onto precast sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% gelatin. After electrophoresis, the gels were washed in 2.5% Triton X-100 twice for 30 minutes to remove all sodium dodecyl sulfate. The gels were then incubated in 50mmol/L TRIS (pH 7.5), 5mmol/L calcium chloride, and 1mmol/L zinc chloride at 37ºC overnight. The gelatin cleavage rate from the photographed gels was analyzed with a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA).

Statistical analysis

The JMP Statistical Discovery Software version 5.1.2 (SAS Institute, Cary, NC, USA) was used to analyze the data. Differences in immunohistochemical expression between groups were analyzed using Fisher’s exact test. Gelatin zymography data were analyzed by one-way ANOVA. P<0.05 was considered to be statistically significant.

Results

Histologic examination of all OLP specimens showed typical findings of this disease, including stratified epithelium, inflammatory subepithelial T lymphocyte infiltration and, occasionally, a sawtooth-like appearance of the epithelium. The infiltrate consisted mainly of leucocytes, which were labeled with leukocyte common antigen (Fig. 1).

Immunohistochemistry results (Table 1) are shown in Fig. 2 for MMP-2 and Fig. 3 for MMP-9. Cells positively stained for MMP-2 were located at epithelial keratinocytes and fibroblasts, and lymphocytes of the subepithelial inflammatory infiltrate. MMP-9-positive cells were located at epithelial keratinocytes and inflammatory infiltrate lymphocytes. There was no difference in MMP-2 expression between the reticular and erosive types of OLP. When all OLP patients were included as one group, the level of MMP-2 in OLP subjects was significantly higher than that in the other two groups (P<0.0001). However, the percentage of positive staining for MMP-9 did not significantly differ among the groups (P=0.5302). MMP-2 and MMP-9 activities in sera were assayed by gelatin zymography. The presence of MMP-2 and MMP-9 was indicated as bands of 72 and 92kDa, respectively, as shown in Fig. 4. The quantitative measurements by the Alphalager 2000 densitometer are shown in Fig. 5. The density of MMP-2 was significantly elevated in the two OLP subgroups, compared with those of the chronic ulcer group and normal control groups (P<0.0001). The levels of MMP-9 in sera, however, did not significantly differ between groups (P=0.1417).

Discussion

The first study investigating the relation of lichen planus and MMPs was reported by Giannelli et al.14

Table 1. Expression of matrix metalloproteinase (MMP)-2/MMP-9 in oral lichen planus (OLP) and control groups

<table>
<thead>
<tr>
<th>Study group</th>
<th>MMP-2 expression*</th>
<th>P</th>
<th>MMP-9 expression*</th>
<th>P</th>
</tr>
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<tr>
<td></td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td></td>
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<tr>
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<tr>
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<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>NOM</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>&lt;0.0001†</td>
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*<25% (+), 26–50% (++) , 51–100% (+++) of the proportion of positively stained cells in tissue sections; †Fisher’s exact test comparing the EOLP and OLP groups combined, the chronic inflammation group and the NOM group. EOLP = erosive oral lichen planus; OLP = reticular and/or plaque oral lichen planus; NOM = normal oral mucosa.

Fig. 1 Leukocytes in an inflamed subepithelial band of an oral lichen planus lesion labeled by leukocyte common antigen using a peroxidase-labeled streptavidin-biotin technique (original magnification ×200).
Fig. 2 Immunohistochemical reactivity for matrix metalloproteinase (MMP)-2 (original magnification ×200). (A) The degree of immunoreactivity to MMP-2 in normal oral mucosa was <25%. (B) The degree of staining in chronic inflammation was 25–50%. The staining pattern of MMP-2 was similar (>50%) among the (C) reticular and/or plaque lesions and (D) erosive lesions.

Fig. 3 Immunohistochemical reactivity for matrix metalloproteinase (MMP)-9 (original magnification ×200). (A) Expression of MMP-9 in normal oral mucosa. (B) Expression of MMP-9 in chronic inflammation. (C) Expression of MMP-9 in reticular oral lichen planus (OLP). (D) Expression of MMP-9 in erosive OLP. The intensity of immunostaining of MMP-9 varied among the four groups, and most of the positively stained cells were located at the subepithelial infiltrating lymphocytes.
Upregulation of MMP-2 in OLP

They reported increased MMP-2 expression in acute stages of lichen planus and suggested that an altered balance between MMP-2 and tissue inhibitors of metalloproteinase-2 may play a role in the destruction of basement membrane. Zhou et al.\textsuperscript{11} also reported MMP-2 expression in OLP epithelium. They suggested that MMPs may act synergistically to degrade the epithelial basement membrane in OLP. Here, we first demonstrated that MMP-2 overexpression in OLP lesions was due to MMP-2 upregulation in circulating plasma. Extracellular proteolysis is generally required for tissue injury in autoimmune and inflammatory diseases, and the plasminogen/plasmin system and MMPs cooperate in such processes.\textsuperscript{15} Thus, basement membrane degradation during OLP progression may be mediated by MMPs.

A previous study showed that elevated expression of inflammatory cytokines, such as interleukin-1\(\alpha\) and tumor necrosis factor (TNF)-\(\alpha\), was found in OLP.\textsuperscript{16} Other studies also showed that MMP synthesis is triggered by tumor necrosis factor-\(\alpha\) and interleukin-1\(\alpha\).\textsuperscript{17,18} Our results indicated that MMP-2 was upregulated in both oral specimens and circulating plasma of OLP patients. MMP-2 overexpression in OLP may be related to inflammatory cytokines. The phenomenon of MMP-2 upregulation in circulation also implies that OLP might be a systemic disorder.

Kim et al.\textsuperscript{19} reported that under upregulation by bone morphogenetic protein (BMP)-4, both MMP-1 and MMP-3 expression in OLP may induce epithelial cells acantholysis and lead to erosive changes. We found that the MMP-2 expression pattern in OLP did not significantly differ between reticular and plaque lesions, and erosive lesions. Thus, the expression of MMP-1 and MMP-3, but not MMP-2 or MMP-9, may be important in determining the phenotype of OLP.

In our clinical experience, some OLP cases were diagnosed by their clinical characterization but failed to match the histopathologic diagnostic criteria. Their pathologic diagnoses were often described as a “chronic ulcer” or “chronic inflammation”. Our study revealed that the MMP-2 level in OLP was significantly higher than that in a chronic ulcer. This finding might provide a diagnostic tool for the differential diagnosis between OLP and chronic ulceration, regardless of whether immunohistochemistry or peripheral serum analysis is used.

In conclusion, we have demonstrated that MMP-2 overexpression in OLP lesions was consistent with MMP-2 upregulation in sera. The MMP-2 level of OLP subjects was significantly higher than that of patients with chronic ulcers and normal controls, not only in lesional sites but also in the circulation. Determination of the MMP-2 level in oral specimens and peripheral blood can be used as a tool for the differential diagnosis between OLP and chronic ulcers. This result also implies that upregulation of MMP-2 might play an important role in the pathogenesis of OLP.

Fig. 4 Gelatin zymogram of matrix metalloproteinase (MMP). Gelatinolytic activity and molecular weight positions of MMP-2 (72 kDa) and MMP-9 (92 kDa) activities are indicated. OLP = oral lichen planus.

Fig. 5 Optical density values of matrix metalloproteinase (MMP)-2 were calculated from their gelatinolytic activities using an Alphalmager 2000 densitometer. The density of MMP-2 was significantly elevated in the two oral lichen planus (OLP) subgroups, compared with those of the chronic ulcer and control groups. *Significantly differs from the control value, at P < 0.05. EOLP = erosive oral lichen planus.
References


