Salivary TNF-α and sCD44 as Markers for Disease Activity and Malignant Transformation in Oral Lichen Planus

Fatheya Zahran, 2Olfat Shaker, 3Dalia Ghalwash, 4Maha Fahmy, 5Mai Mostafa, 1Safia Al-Attas

1Department of Oral Diagnostic Sciences, Faculty of Dentistry, King Abdulaziz University, Jeddah, Saudi Arabia
2Department of Medical Biochemistry, Faculty of Medicine, Cairo University, Cairo, Egypt.
3Department of Oral Medicine & Periodontology, MSA University, 6th October City, Egypt.
4Department of Oral Medicine & Periodontology, MHI University, Cairo, Egypt.

ABSTRACT

Background: Testing the validity of utilizing salivary markers as monitors for disease activity, therapeutic progress and potential malignant transformation of oral lichen planus (OLP) lesions. Materials and Methods: Seventy two subjects were recruited and divided into 3 groups according to clinical presentation. Group I: 45 OLP patients, no histopathologic signs of dysplasia; 15 reticulated type and 30 with symptomatic OLP (15 atrophic and 15 erosive types); group II: 12 OLP patients with dysplastic changes and group III: 15 controls free from oral mucosal disease. Biopsy was carried out for group I before and after corticosteroid administration and from surgical sites in controls. All individuals provided whole unstimulated salivary specimens. TNF-α levels were determined in tissue and salivary specimens of groups I and III, utilizing R&D system ELISA kits (Mineapolis, USA). Salivary total soluble (s) CD44 was estimated in all groups, utilizing an ELISA assay (Bender MedSystems, Vienna, Austria). Results: Oral lichen planus patients showed highly significant increase in levels of both investigated markers, compared to healthy controls, whether in tissues or saliva. After corticosteroid administration, TNF-α level in oral lichen planus patients dropped significantly simultaneously in tissues and saliva, however, they were still higher than the controls. Levels of salivary sCD44 were significantly higher in group II than groups I & III: with evident correlation between values above 19.2 ng/ml and presence of dysplastic changes. Conclusion: Salivary TNF-α level might furnish a reliable marker for OLP disease activity and therapeutic progress, while salivary sCD44 could be considered a valuable, reliable, non-invasive marker for dysplastic changes in OLP.

INTRODUCTION

Oral Lichen planus (OLP) is a chronic inflammatory autoimmune disease[1]. According to Payeras et al. [2], OLP is a T- cell mediated reaction towards an unknown trigger. The disease affects the middle-aged female population twice more than the male [1]. Worldwide the reported prevalence rates range from 0.2 to 2% [3]. However, in the Arab population the prevalence ranges from 0.35% to 1.7% [4]. Clinically it is presented as cycle of remissions and exacerbations of 6 clinical forms 1.Reticular, 2.Popular, 3.Plaque-like, 4.Atrophic or erythematous, 5.Erosive or ulcerative, 6.Bullous[1, 5]. Up to date, OLP is treated symptomatically and there is no effective treatment to cure the disease permanently [1, 6]. Corticosteroids remain the mainstay treatment for OLP, and the benefit is related to their immunosuppressive and anti-inflammatory properties [7, 8]. The World Health Organization (WHO) classified OLP as one of the potentially malignant oral disorders because of the 0.4%-to 5.3% reported rates of malignant transformation with more than 5 years follow up duration [9]. All clinical types of OLP showed malignant transformation risk [10, 11]. Histological study with all its drawbacks still remains the gold standard for determining the malignant transformation risk in OLP [9, 12]. Thus, there is an urgent need of objective biomarkers that assess the risk of OLP malignant transformations without substantial inter observer and intra observer variations [9]. The role of oral salivary fluids in the integrity and maintenance of oral cavity is unquestionable. Nowadays, the uses of salivary oral fluids for oral and systemic diseases...
diagnosis and/or monitoring are promising. The components within salivary fluids provide clues to local and systemic diseases [13, 14]. Several authors have suggested different salivary biomarkers for OLP diagnosis, treatment and prognosis monitoring [13, 15-21]. However, the data seems inconsistent [14]. The salivary cytokine tumor necrosis factor alpha (TNF-α), has been found to be increased in patients with cancer and premalignant lesions such as leukoplakia [15, 22]. It has been also implicated to play a role in OLP initiation and progression, as it is highly detected in the sera and local tissues of OLP patients compared to the control subjects [23]. Additionally, increased levels of cells with mRNA for TNF-α have been found in all OLP lesions [24]. Pezelj-Ribaric et al. [25] postulated that enhanced salivary TNF-α production correlates with OLP severity. Moreover, they suggested that the persistence of high salivary TNF-α could contribute to malignant transformation. On the other hand, many authors over the last decade studied a cellular protein called CD44 in relation to carcinogenesis. The CD44 glycoprotein is an adhesion molecule of the hyaluronic receptor family. It is altered during inflammatory responses and cellular malfunctioning during tumor progression. Tumors of epithelial origin express CD44 in multiple isoforms or variants; some isoforms are related to specific cancer cells. However, there is still uncertainty regarding the exact mechanism by which CD44 participates in growth of cancer or the inflammatory response [26]. Up to our knowledge no previous study has investigated the prognostic value of salivary sCD44 in OLP.

The aim of the research is to assess the diagnostic and/or the prognostic function of salivary TNF-α and sCD44 in oral lichen planus.

MATERIALS AND METHODS

The approval to conduct the research was obtained from the ethical committee of the faculty of Dentistry, King Abdulaziz University, Jeddah. Seventy two subjects were recruited and divided into 3 groups according to clinical presentation. Group I: comprised 45 patients with OLP, no histopathologic signs of dysplasia, 15 reticular type and 30 patients with symptomatic OLP (15 atrophic and 15 erosive types), group II: 12 OLP cases showing dysplastic changes and group III: 15 control subjects with no oral mucosal disease. All patients signed a written consent.

All the selected patients fulfilled the following criteria: Patients included in this study were free from any systemic diseases. No history of drugs for at least six months prior to the initiation of the study. Patients who had been taking drugs capable of inducing lichenoid reactions were excluded. They were free from severe periodontal disease.

All the selected control subjects fulfilled the following criteria: Were free from any systemic diseases. They were age and gender matching to the included OLP patients. They were free from severe periodontal disease.

Treatment regimen used for symptomatic OLP cases: The 30 patients suffering from atrophic and erosive OLP were treated according to the severity of their lesions, either with topical or systemic corticosteroids, according to the condition until clinical remission was observed. Topically, patients received treatment in the form of Triamcinolone acetonide (0.1% in orabase). Patients were advised to apply the drug topically on the lesions, 4 times a day i.e., following each meal and at bed time, for 4 weeks [27]. Patients needing systemic corticosteroids were instructed to take 40 mg prednisone orally, 1.5 hours after waking up as a single daily dose for 14 consecutive days followed by a gradual taper for 2 weeks, according to Gorsky et al. [28]. All patients were followed up throughout the treatment period and therapy was prolonged if signs and symptoms did not disappear.

Assessment of symptoms: disappearance of pain (or discomfort) experienced by patients was taken as indicator for achieving remission. It was measured by the visual analogue scale (VAS) [29], where the patients were asked to mark a 10 cm line at a level equivalent to the magnitude of their pain.

Biopsy:

Biopsy specimens were taken from group I: only once from reticular OLP cases and from symptomatic patients at the initial diagnosis and after steroid therapy and from group II only at time of diagnosis to:
1. Ascertain the diagnosis and detect presence of dysplasia that might indicate early malignant transformation.
2. Provide tissue specimen for detection of TNF-α levels before and after treatment.

Biopsy specimens were also obtained from group III to act as control group. Tissues were obtained during surgical procedures the individuals were already subjected to, such as frenectomy or surgical extraction of impacted teeth. A surgical double wedge incisional biopsy was carried out to a depth of about 2 mm. Then, specimens were each halved into 2 pieces:
1. For histopathologic examination. Biopsy specimens were immersed in 10% neutral buffered formalin and then paraffin blocks were prepared. Five microns thick sections were cut and stained with conventional Hematoxylin & Eosin.
2. For detection of TNF-α levels (only groups I and III). Tissue specimens were homogenized in 200 μl of phosphate buffer saline. Centrifugation of the homogenate was carried out for 2 minutes at 10,000 xg and a 0.45 μm low protein binding membrane was used for supernatant filtration, which was then divided into 0.5 ml aliquots that were kept at -80°C till further processing.
Salivary specimens' collection:
Whole unstimulated saliva (WUS) was collected according to the methodology of Navazesh[30]. After collection, all samples were immediately stored at -80°C until assayed.

TNF-α Assay:
Concentrations of TNF-α in both saliva and tissue samples were estimated by human TNF-α/ TNF SFIA immunoassay ELISA kit (Quantikine, R&D Systems, Inc., Minneapolis, USA). All reagents and working standards were prepared, and then the assay was performed according to manufacturer’s instructions. Results were expressed in pg/ml for saliva and pg/mg for tissues.

Detection of Soluble CD44 level in salivary samples:
Salivary total soluble (s)CD44 was investigated in all groups. Levels of soluble CD44 (sCD44) were measured using an ELISA assay (Bender MedSystems, Vienna, Austria) that identifies all normal and variant isoforms (total sCD44).

Saliva samples were centrifuged at 2000 Xg and the supernatants were separated and stored at -80°C till processing, which was done according to manufacturer’s instructions. Results were expressed in ng/ml.

Statistical Analysis:
Student’s t-test was used to compare between mean levels of tissue and salivary TNF-α as well as salivary sCD44 between different groups.

Results:
The present study was carried out on 72 subjects, 45 OLP patients without dysplasia (15 patients suffering from reticular type of oral lichen planus, 15 patients suffering from erosive type, 15 patients suffering from atrophic type), 12 with OLP showing histopathologic signs of dysplasia and 15 healthy control persons. Descriptive data of all included OLP patients are shown in table (1). TNF-α levels were detected in the salivary and tissue specimens of all OLP patients. The levels registered in the 45 OLP patients without dysplasia were compared with that of the 15 unaffected controls, without treatment in reticular type, before and after treatment with corticosteroids in cases with the erosive and the atrophic types. The levels varied significantly among the studied groups. Table (2) shows the highly significant statistical difference (P< 0.01) in salivary and tissue TNF-α level when any of the OLP subgroups (reticular, atrophic, and erosive) was compared to the healthy control group. Also when atrophic and erosive OLP cases were compared to the reticular form subgroup, a highly significant statistical difference (P< 0.01) was revealed. Table (3) shows the salivary and tissue TNF-α levels in atrophic and erosive OLP cases after treatment for 4 weeks with systemic corticosteroids, compared to the control values. As shown by the table, despite the drop in the levels of salivary and tissue TNF-α levels in OLP cases after treatment, they are still higher than the control levels with a highly significant statistical difference (P< 0.01). In figure (1) it can be seen that there was a highly significant drop in salivary TNF-α mean levels after treatment (Student-t test showed P- value <0.01). The values of treated cases approached the values for reticular OLP (P> 0.5). The figure illustrates the results shown by table (3), where it shows the persistent highly significant difference between the treated OLP cases and the controls. Figure (2) illustrates the tissue TNF-α mean level in studied groups. The figure shows that after 4 weeks of corticosteroid therapy, a highly significant drop in their values occurred (Student-t test showed P- value <0.01), their values approached the reticular form levels (Student-t test showed P- value > 0.05), but they were still statistically significantly higher than the control levels as previously shown in table (2). Regarding sCD44, table (4) summarizes the obtained results, where the salivary levels in OLP cases with dysplasia registered a highly significant increase compared to either healthy controls or OLP cases without dysplasia (Student-t test showed P- value <0.01). On the other hand, the cases without dysplasia showed a statistically insignificant difference when compared to healthy controls.

Table 1: Demographic data of OLP patients and normal control group.

<table>
<thead>
<tr>
<th>Gender</th>
<th>OLP without dysplasia</th>
<th>OLP with Dysplasia</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reticular (15)</td>
<td>Atrophic (15)</td>
<td>Erosive (15)</td>
</tr>
<tr>
<td>Male</td>
<td>8 (53.33%)</td>
<td>4 (26.67%)</td>
<td>7 (46.67%)</td>
</tr>
<tr>
<td>Female</td>
<td>7 (46.67%)</td>
<td>11 (73.33%)</td>
<td>8 (53.33%)</td>
</tr>
<tr>
<td>Age</td>
<td>Range</td>
<td>40-70</td>
<td>40-90</td>
</tr>
<tr>
<td>Mean ±SD</td>
<td>46.7±5.2</td>
<td>56.6±6.55</td>
<td>47.0±10.55</td>
</tr>
<tr>
<td>Smoking</td>
<td>Smoker</td>
<td>7 (46.67%)</td>
<td>0</td>
</tr>
<tr>
<td>Non smoker</td>
<td>7 (46.67%)</td>
<td>0</td>
<td>15 (100%)</td>
</tr>
</tbody>
</table>
Table 2: Statistical analysis for salivary and tissue TNF-α levels among patients before treatment compared to control group (Student-t test).

<table>
<thead>
<tr>
<th></th>
<th>Salivary TNF-α (pg/ml)</th>
<th>Tissue TNF-α (pg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before treatment</td>
<td>Control (n=15)</td>
</tr>
<tr>
<td>Reticular (n=15)</td>
<td>Mean 5.55</td>
<td>SD 0.91</td>
</tr>
<tr>
<td>Atrophic (n=15)</td>
<td>22.34</td>
<td>5.91</td>
</tr>
<tr>
<td>Erosive (n=15)</td>
<td>14.53</td>
<td>2.84</td>
</tr>
</tbody>
</table>

Table 3: Statistical analysis for salivary and tissue TNF-α levels among patients after treatment compared to control group (Student-t test).

<table>
<thead>
<tr>
<th></th>
<th>Salivary TNF-α (pg/ml)</th>
<th>Tissue TNF-α (pg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After treatment</td>
<td>Control (n=15)</td>
</tr>
<tr>
<td>Reticular (n=15)</td>
<td>Non applicable</td>
<td>1.952</td>
</tr>
<tr>
<td>Atrophic (n=15)</td>
<td>10.36</td>
<td>2.08</td>
</tr>
<tr>
<td>Erosive (n=15)</td>
<td>6.03</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Fig. 1: Salivary TNF-α (pg/ml) mean values in OLP subgroups before and after treatment compared to each other and control group.

Fig. 2: Tissue TNF-α (pg/mg) mean values in OLP subgroups before and after treatment compared to each other and control group.

Table 4: Statistical analysis for salivary sCD44 levels (ng/ml) among OLP patients with and without dysplasia compared to control group (Student-t test).

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>P-value Vs control</th>
<th>P-value Vs OLP without dysplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=15)</td>
<td>12.37</td>
<td>3.82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OLP without dysplasia</td>
<td>14.67</td>
<td>7.27</td>
<td>0.388 (&gt;0.05)</td>
<td></td>
</tr>
</tbody>
</table>
Saliva collection is a simple and noninvasive procedure, suggested to be used as an aid in disease diagnosis and monitoring [14]. Its attractions for diagnosis are increased by the commercial availability of some easily used diagnostic tests [31]. Very early, Pezelj-Ribaric et al. [25] pointed out the importance and availability of salivary analysis for cytokine levels particularly TNF-α in monitoring disease activity in OLP cases. Based on these data, Rhoduset et al. [32] encouraged the use of salivary cytokine levels, specifically TNF-α, IL-1α, IL-6 and IL-8, to monitor the corticosteroid therapeutic outcome in OLP patients. On the other hand, detecting alterations in expression and structure of CD44 have been associated with several types of cancers [33]. Consequently, the current study has been carried out to explore the value of detecting salivary TNF-α and CD44 level in assessing the disease prognosis as well as treatment monitoring. The control group subjects were chosen to have matching periodontal status as OLP patients to avoid any misinterpretation of results due to changes in TNF-α level due to periodontal disease [34]. They were also matched regarding age and gender to avoid any changes due to such variables [25]. In order to estimate TNF-α level during total disease remission, corticosteroids, which are known to be the mainstay for OLP therapy, were utilized in the present study for symptomatic cases (i.e. Erosive and atrophic groups). However, no specific regimen was stiffly applied to OLP patients. Instead, both topical and systemic corticosteroids were used, according to the clinical severity and the timing was controlled by the patients’ going through remission [34, 35]. To determine TNF-α level, ELISA kit was used, because it is the most recommended simple, sensitive and inexpensive test [36, 37]. The obtained results showed that TNF-α was detectable in salivary and tissue samples of all studied individuals; however its levels registered highly significant increase in saliva and tissues in all OLP patients when compared to the control group. Among OLP patients, the atrophic type, followed by the erosive (before treatment) showed the highest tissue and salivary values, and even after reaching clinical remission, both groups still showed levels with highly significant increase compared to the control group. The mean salivary TNF-α in the atrophic group was even still higher after corticosteroid therapy than the reticular group. Thus, clinical improvement of oral lichen planus lesions after treatment with corticosteroid therapy was accompanied by significant simultaneous decrease in tissue and salivary TNF-α level; however normal control levels were not reached. The inhibitory effect of TNF-α on the proliferation of keratinocytes and which was documented by Weedon [38], could explain the relation between the highest increase in TNF-α level and the atrophic OLP lesions. Actually, stem cell damage and epithelial atrophy were associated with sustained production of TNF-α [39]. Our results agreed with Pezelj-Ribaric et al. [25] and Rhoduset et al. [32] who deduced that salivary TNF-α analysis provided a perfect image for tissue levels of cytokines in OLP cases, and could be utilized in monitoring the disease activity status. Rhoduset et al. [32] registered a significant lowering in salivary TNF-α level with topical corticosteroid therapy. They considered their results as first evidence for the efficacy of salivary cytokine level determination as a monitor for outcome in OLP. Subsequently, Ghallab et al. [18] reported similar findings with the erosive forms of OLP. The present results confirm such conclusions, where the treated groups (erosive and atrophic) showed a highly significant decrease in salivary TNF-α after corticosteroid therapy. Interestingly, TNF-α level after corticosteroid therapy even with disease remission, was higher significantly than control level, and the atrophic group was higher than the untreated reticular OLP patients. This sustained salivary TNF-α, despite clinical remission could imply the basis for disease chronicity [25]. Some previous authors even went further in explaining the sustained levels of cytokines to the possibility of indicating malignant changes in OLP. They then proposed the potential prognostic value of salivary TNF-α [15]. Actually, the erosive and atrophic forms of OLP are those mostly incriminated with the potential of malignant transformation [40]. The low expression of TNF-α in both the salivary and tissue samples of the control group in contrast to OLP patients, may confirm the TNF-α role in the pathogenesis of OLP. Also what assured this theory is the decrease of TNF-α after OLP treatment. Similarly, Zhou et al. [41] reported significant increase of TNF-α level in OLP patients than the controls using ELISA as well as Sklavounou et al. [42] who found significant expression of TNF-α throughout OLP epithelium compared to controls. Therefore, we agreed with them regarding the crucial role played by TNF-α in OLP pathogenesis. Moreover, the presence of TNF-α in the oral salivary fluids and the close resemblance of the fluctuations in its salivary levels to the changes in tissue levels makes salivary analysis a promising aid in diagnosing and monitoring OLP, in particular the severe types. Besides TNF-α,salivary sCD44 was chosen because a previous study by Ghalwashet al. [43] revealed that oral cancer patients showed significant raise in sCD44 level with a cut-off point value of 19.2 - 20 ng/ml with sensitivity and specificity 100% and 67%, respectively, and they recommended it as a reliable molecular marker for malignant transformation in the oral cavity. They also reported a good correlation between the sCD44 levels and the grading as well as the aggressiveness of malignancy. Here we also used ELISA assay (Bender MedSystems) to measure the salivary sCD44, as it measured all normal CD44 and variant isofoms. The specificity of the assay has been previously confirmed by Western blot [44]. The present results showed thatsalivary sCD44 was detected in all OLP patients

| OLP with dysplasia (n=12) | 34.98 | 11.27 | 0.000 (< 0.01) | 0.008 (< 0.01) |

**Discussion:**

Saliva collection is a simple and noninvasive procedure, suggested to be used as an aid in disease diagnosis and monitoring [14]. Its attractions for diagnosis are increased by the commercial availability of some easily used diagnostic tests [31]. Very early, Pezelj-Ribaric et al. [25] pointed out the importance and availability of salivary analysis for cytokine levels particularly TNF-α in monitoring disease activity in OLP cases. Based on these data, Rhoduset et al. [32] encouraged the use of salivary cytokine levels, specifically TNF-α, IL-1 α, IL-6 and IL-8, to monitor the corticosteroid therapeutic outcome in OLP patients. On the other hand, detecting alterations in expression and structure of CD44 have been associated with several types of cancers [33]. Consequently, the current study has been carried out to explore the value of detecting salivary TNF-α and CD44 level in assessing the disease prognosis as well as treatment monitoring. 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and it was more elevated than that of the control subjects with a highly significant difference. Moreover, comparison between OLP patients, with and without dysplasia, revealed that the dysplastic group had significantly higher levels of salivary sCD44 than the non-dysplastic group (P< 0.01). Our finding is in accordance with Chaiyarat et al. [17], who also demonstrated significant elevations in the salivary sCD44 levels in OLP patients than the healthy controls. The investigators claimed the presence of chronic inflammation in OLP lesions as the cause of the increase in salivary sCD44 levels. Perhaps the lower values shown by non-dysplastic OLP lesions could be the result of inflammatory reaction. However, lesions with dysplasia showed higher sCD44 levels, all above 19.2ng/ml, which was in accordance with the previous results of Ghalwash et al. [43] while the non-dysplastic lesions registered values, all below the 19.2ng/ml value, which confirms this value as a cutoff point between dysplastic lesions and those without dysplasia. Based on the detected significant raises in salivary sCD44 levels in OLP patients with dysplasia, we can consider it a potential marker for malignant transformation of these lesions. Thus, it may be used as a diagnostic tool. However, the prognostic value of CD44 in head and neck squamous cell cancer still remains controversial [45]. Therefore, more future studies with larger sample size are needed to accept or reject the idea of using such markers in predicting malignant transformation in OLP.

Conclusions:

Salivary markers in cases with OLP seem to act as a mirror for pathologic changes in tissues, where they can monitor disease severity during exacerbations and decrease in the inflammatory mediators in remissions, as well as effect of treatment and dysplastic changes. Salivary TNF-α level conforms perfectly to the disease severity and can monitor the treatment progress.

The present study adds evidence to the crucial role that TNF-α seems to have in the immune-pathogenesis of OLP and which could be inhibited by the use of corticosteroids. However, such inhibition does not seem to be sufficient to return its levels back to normal, which results in disease chronicity. This points out to the importance of searching for new therapeutic modalities that might target TNF-α more specifically. The results also revealed a correlation between OLP with dysplasia and salivary sCD44 levels above 19.2 ng/ml. Future studies on larger samples are required to validate and generalize the present results.

Conflict Of Interest:

The authors declare that no competing financial interests exist and no conflict of interest.

Authors’ Contribution and Previous Presentation:

The authors have all contributed equally to the work. The preliminary results (with smaller sample size) were presented orally in the 101st FDI Annual World Dental Congress, Istanbul, Turkey on 28th August, 2013 by Prof. Fat’heya M. Zahran. Data was collected for these preliminary results by Dalia M.Ghalwash, Maha A. Fahmy and Mai A. Mostafa, under the supervision of Prof. Fat’heya M. Zahran, while Prof. Olfa G. Shaker carried out lab work. Afterwards, the sample size was increased, final results analyzed and manuscript prepared and critically revised. These last steps were carried out by Prof. Fat’heya Zahran and Dr. Safia Al-Attas.

REFERENCES


