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Research Article

Study of HSP27 expression in two types of oral lichen planus.

Running title: HSP27 and OLP

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ABSTRACT

Introduction: Oral lichen planus (OLP) is a chronic inflammatory disease with unknown etiology. HSP27 has been involved in pathogenesis of chronic diseases. The aim of this study was to evaluate HSP27 expression in two subtypes of OLP in comparison with normal mucosa.

Methods and Materials: In this cross-sectional research, study group included 51 samples of reticular and erosive lichen planus and normal mucosa (NM). Tissue sections were stained with HSP27 antibody using immunohistochemistry. Percentage of stained cells, staining intensity, expression site in epithelium and cellular localization was compared between studied groups. $P<0.05$ were considered significant.

Results: Comparison of stained cells in basal layer showed significant decrease in HSP27 expression in reticular OLP compared to NM but this comparison did not show significant difference between erosive OLP and NM and also between reticular and erosive OLP. Suprabasal and superficial layers showed significant decrease of HSP27 expression in reticular OLP compared to NM and in reticular OLP compared to erosive type. Comparison of expression site and cellular localization did not show any significant difference between studied groups.

Conclusion: Decrease of HSP27 expression in reticular OLP compared to NM suggests its involvement in pathogenesis of this type of OLP.

Keywords: Heat shock proteins, Oral lichen planus, HSP27

INTRODUCTION

Oral lichen planus (OLP) is a common chronic inflammatory disease of oral mucosa with unknown etiology. (1) It is believed that either an autoimmune response to basal cell antigens or a hyperimmune response to common antigens between microbial agents or basal cells cause this disease. (2)

Heat shock proteins (HSPs) are a set of cellular protective proteins that produce usually in response to heat shock and other stresses such as infection, inflammation and etc. and play the

major role in protecting cells against damage in stressful circumstances. (3,4)

HSPs may play a role in autoimmune diseases; It is believed that overexpression of self HSPs or cross reaction of pathogens' HSP with host's HSPs are involved in autoimmune or hyperimmune responses. (2, 3, 5)

It is not clear that whether there is a role for HSPs in initiation or persistence of lymphocytic inflammatory response which occurs in pathogenesis of oral lichen planus or not (5). Because lichen planus appears a T-cell mediated autoimmune or hyperimmune circumstance, HSPs may have a role in pathogenesis of this disease. (3)

On the other hand, keratinocyte's HSP expression in lichen planus may be a secondary process which is associated with preexisting inflammation. (1) HSPs may help to persistence or chronicity of oral lichen planus or if present, they can just be reflective of cellular damage. (3)

HSP27 belongs to small HSPs family which overexpresses during cellular differentiation and development, normal reparative processes and in inflammatory response; It has been also involved in pathogenesis of chronic diseases which probably play a protective role. (5)

According to little and contradictory available data regarding HSP27 expression in oral lichen planus and described role for HSP27 during inflammation and pathogenesis of chronic diseases and also autoimmune or hyperimmune circumstances, the aim of present study was to evaluate HSP27 expression in two subtypes of oral lichen planus in comparison to normal oral mucosa.

METHODS AND MATERIALS

This cross-sectional analytical, descriptional study included 34 samples of two subtypes of oral lichen planus (including 17 cases of reticular OLP and 17 cases of erosive OLP) and 17 samples of normal oral mucosa or NOM (normal gingival tissues obtained from crown lengthening surgical procedure with clinically and histopathologically absent or minimal inflammation as control group) which were retrieved from paraffin-embedded tissue blocks of archive of our oral and maxillofacial pathology department.

Four-micron sections were prepared from each tissue blocks and stained with hematoxylin and eosin method. Microscopic slides were reviewed to verify the diagnosis and clinicohistopathologic subtype of lichen planus (reticular and erosive subtypes) was determined. Criteria for classification of these two subtypes were based on Neville et al. (6) and Greenberg et al. (7). Specimens that were clinically suspicious to lichen planus by oral medicine specialist and were histopathologically proved to be lichen planus by pathologist were included in the study. Specimens whose diagnosis was inconsistent with the initial diagnosis were excluded from the study. Specimens were classified as reticular subtype with clinical characteristics of Wickham's striae without atrophic-erosive areas and with histopathologic characteristics of lichen planus (e.g. hydropic degeneration of basal cells) without atrophic-erosive epithelium (with normal or hyperplastic epithelium). Specimens were classified as erosive subtype with clinical characteristics of Wickham's striae at the periphery and presence of atrophic-erosive areas and with histopathologic characteristics of lichen planus (e.g. hydropic degeneration of basal cells) with atrophic-erosive epithelium. Specimens were included in the study that the patient had not received any treatment for his or her disease. Furthermore, tissue blocks that their tissue were insufficient for analysis or had improper quality or fixation were excluded from the study.

Thereafter, another 3 micron sections were prepared from paraffin-embedded tissue blocks and stained with HSP27 antibody ((Novocastra TM Liquid Mouse Monoclonal Antibody HSP27; Leica Biosystems, Newcastle, United Kingdom, Product Code: HSP27 NCL-HSP27, Clone: RJT24, Ig Class: IgG2b) using immunohistochemistry. Normal gastric mucosa was used as positive control and omission of

primary antibody were used as negative control. (5)

At immunohistochemical staining, tissue sections were immersed in citrate buffer 10 Mm (PH=6) and were heated 5 minutes in oven microwave at 95°C for antigen retrieval. 3% hydrogen peroxide solution were poured on slides for blockage of endogenous peroxidase activity. Primary antibody bonding was detected using streptavidin-biotin immunoperoxidase method and diaminobenzidin was used as chromogen. (5)

Stained slides were assessed by pathologist using Olympus CX21 light microscope (Olympus Corporation, Tokyo, Japan) at ×100 and ×400 magnifications. At this assessment, percentage of stained cells, staining intensity, expression site in epithelium (basal, suprabasal and superficial) and cellular localization (cytoplasmic, nuclear and membranous) were considered.

Five microscopic fields were selected as hot spots (fields on which epithelial cells had greatest staining) at ×100 magnification and percentage of stained cells were calculated at ×400 magnification.

Three areas of epithelium (basal, suprabasal and superficial) were analyzed in each sample and percentage of stained cells were semi-quantitatively categorized as follow:

1: percentage of stained cells <50%; 2: 50%≤ percentage of stained cells≤69%; 3: percentage of stained cells≥70%. (5)

Staining intensity for HSP27 immunomarker at the above-mentioned areas were categorized into negative (-), mild (+), moderate (++) and sever (+++). (5)

Cellular localization of HSP27 immunomarker was also evaluated and categorized as cytoplasmic, nuclear or membranous. (5)

Data were analyzed with SPSS 21 statistical software using Chi-square and t-test statistical tests. P-value<0.05 was considered significant.

Ethical Approvals

The study has been independently reviewed and approved by ethical board of our university (Code: MUBABOL.REC.1396.84)

RESULTS

In this study, 51 histopathological slides (15 reticular OLP, 15 erosive OLP and 15 normal mucosa) were investigated.

From 34 OLP samples, 22 specimens belonged to females and 12 specimens to males. Mean age of patients was 47 years old. 28 OLPs specimens located at buccal mucosa, 4 specimens at tongue mucosa and 2 specimens at lip mucosa.

Based on table 1, most of specimens in OLP and NOM groups were located in category 3 (percentage of stained cells ≥70%). In basal and superficial layers, there were not any significant differences between OLP and NOM groups in the light of percentage of stained cells for HSP27 (P-value>0.05). In suprabasal layer, there was significant difference between OLP and NOM groups in the light of percentage of stained cells for HSP27 (P-value<0.05).

Based on table 2, all of NOM samples had moderate (++) to severe (+++) staining intensity in all basal, suprabasal and superficial layers but in OLP group, some of the specimens had mild (+) or even absent (in superficial layer) staining intensity. There were significant differences between OLP and NOM groups in all basal, suprabasal and superficial layers in the light of staining intensity (P-values<0.05)

HSP27 expression was observed an all NOM samples at all three basal, suprabasal and superficial layers but among OLP samples, some of them did not show hsp27 expression at superficial layer. There wasn't any significant difference between these two groups in the light of Hsp27 expression site in epithelium (P-value=0.71>0.05)

None of the OLP and NOM samples had Hsp27 nuclear expression. HSP27 cytoplasmic expression was observed in all OLP and NOM samples. Some of the OLP and NOM samples showed membranous staining. No significant difference was seen between these two groups in the light of Hsp27 cellular localization. (P-value=0.06>0.05)

Based on table 3, in basal, suprabasal and superficial layers, there were significant differences between reticular OLP and NOM groups in the light of percentage of stained cells for HSP27 (P-value<0.05). In erosive OLP like NOM group, the majority of samples located in the category 3 (percentage of stained cells≥70%) at all three basal, suprabasal and superficial layers. At basal, suprabasal and superficial layers, there weren't any significant differences between erosive OLP and NOM groups in the light of percentage of stained cells for HSP27 (P-value>0.05). At basal layer, there wasn't any significant difference between erosive and reticular OLP in the light of percentage of stained cells for HSP27 (P-value=0.28>0.05), but there were significant differences between these two subtypes at suprabasal and superficial layers (P-value=0.003 and 0.01 respectively)

Based on table 4, in reticular lichen planus, at basal and suprabasal layers, the majority of samples had mild (+) staining intensity and 6 samples had no staining for HSP27 at superficial layer. There were significant differences between reticular OLP and NOM groups at basal, suprabasal and superficial layers in the light of staining intensity (P-values<0.05). In erosive OLP, at basal, suprabasal and superficial layers, the majority of samples had moderate (++) to severe (+++) staining intensity. There weren't any significant differences between erosive OLP and NOM groups at basal, suprabasal and superficial layers in the light of staining intensity for HSP27 (P-values>0.05). At superficial layer, there was not any significant difference between reticular and erosive OLP in the light of staining intensity for HSP27 (P-value=0.051>0.05), but at basal and suprabasal layers, there were significant differences between these two groups (P-value= 0.002 and <0.001, respectively).

All of NOM samples showed HSP27 expression at basal, suprabasal and superficial layers. Some of the reticular OLP did not show HSP27 expression at superficial layer. There was not any significant difference between reticular OLP and NOM groups in the light of expression site of HSP27 at epithelium. (P-value=0.63>0.05). Only 3 samples of the erosive OLP didn't show HSP27 expression at superficial layer. No significant difference was found between erosive OLP and NOM groups in the light of expression site of HSP27 at epithelium (P-value=0.90>0.05). There was not any significant difference between erosive and reticular OLP in the light of expression site of HSP27 at epithelium (basal, suprabasal and superficial). (P-value=0.87>0.05)

All of reticular OLP and NOM samples had HSP27 cytoplasmic expression. Although number of samples with membranous expression was more in NOM group than reticular OLP (13 versus 5), altogether there was no significant difference between reticular OLP and NOM groups in the light of cellular localization of HSP27. (P-value=0.12>0.05) All of erosive OLP had HSP27 cytoplasmic expression. Although number of samples with membranous expression was more in NOM group than erosive OLP (13 versus 5), altogether there was no significant difference between erosive OLP and NOM groups in the light of cellular localization of HSP27 (P-value=0.12>0.05). No significant difference was found between erosive and reticular OLP in the light of cellular localization (cytoplasmic, nuclear and membranous) of HSP27. (P-value=1.00>0.05)

DISCUSSION

According to what mentioned in introduction, HSP27 has been involved in pathogenesis of chronic diseases. Therefore, the aim of the present study was to evaluate HSP27 expression in two subtypes of OLP in comparison with normal mucosa.

In our study, at suprabasal layer, there was significant difference between OLP and NOM in the light of percentage of stained cells for HSP27 and percentage of stained cells was significantly lower in OLPs than NOM group. This finding supports HSP27 involvement in OLP pathogenesis that appears as difference in percentage of stained cells at suprabasal layer. Changes in HSP27

expression (downregulation of HSP27 at suprabasal layer) in epithelium of OLP patients may related to pathologic stress such as chronic inflammation in OLP patients.

In our study, at all three basal, suprabasal and superficial layers, there were significant differences between OLP and NOM in the light of staining intensity for HSP27 and OLPs had significantly weaker staining intensity than NOMs at all three layers. Another time, this finding supports HSP27 involvement in OLP pathogenesis that appears as difference in staining intensity at all three layers. Therefore, according to our study, HSP27 involvement in OLP pathogenesis appears as difference in percentage of stained cells at suprabasal layer and difference of staining intensity at all three basal, suprabasal and superficial layers.

In our study, at basal, suprabasal and superficial layers, there were significant differences between reticular OLP and NOM groups in the light of percentage of stained cells for HSP27 and percentage of stained cells was significantly lower at all three layers in reticular OLP than NOM. This finding suggests HSP27 involvement in reticular OLP pathogenesis that appears as decrease in percentage of stained cells at all three studied layers in reticular OLP. Changes in HSP27 expression (downregulation of HSP27 at all three studied layers) in epithelium of reticular OLP patients may be related to pathologic stress such as chronic inflammation in reticular OLP patients.

In our study, at all three basal, suprabasal and superficial layers, there were significant differences between reticular OLP and NOM in the light of staining intensity for HSP27 and reticular OLPs had significantly weaker staining intensity than NOMs at all three layers. Another time, this finding supports HSP27 involvement in reticular OLP pathogenesis that appears as decrease in staining intensity at all three layers. Therefore, according to our study, HSP27 involvement in OLP pathogenesis appears as difference in percentage of stained cells at suprabasal layer and difference of staining intensity at all three basal, suprabasal and superficial layers.

In our study, at basal, suprabasal and superficial layers, there were no significant differences between erosive OLP and NOM groups in the light of percentage of stained cells for HSP27. This finding suggests that HSP27 do not involve in pathogenesis of erosive OLP. Expressed HSP27s in erosive OLP can help to persistence or chronicity of disease or can only be reflective of cellular damage.

In our study, at all three basal, suprabasal and superficial layers, no significant differences were observed between erosive OLP and NOM in the light of staining intensity for HSP27. Another time, this finding supports lack of HSP27 involvement in erosive OLP pathogenesis.

According to our study, there were not significant differences between erosive OLP and NOM in the light of HSP27 expression site at epithelium (basal, suprabasal and superficial) and HSP27 cellular localization (cytoplasmic, nuclear or membranous) that another time emphasize lack of HSP27 involvement in erosive OLP pathogenesis.

In our study, there were significant differences between reticular and erosive OLP at suprabasal and superficial layers in the light of percentage of stained cells for HSP27such that percentage of stained cells was significantly lower in reticular OLP. Presence of this difference reminds different pathogenesis of these two subtypes of OLP and suggest different role of HSP27 in reticular and erosive OLP pathogenesis.

Based on our study, there were significant differences between reticular and erosive OLP at basal and suprabasal layers in the light of HSP27 staining intensity such that staining intensity was significantly weaker in reticular OLP. Presence of this difference reminds different pathogenesis of these two subtypes of OLP and suggest different role of HSP27 in reticular and erosive OLP pathogenesis.

In Bramanti TE et al. study (3), in six OLPs, basal keratinocytes stained intensely for HSP27 while control samples showed only mild staining that is

to some extent in contradiction with our results because in our study, intense staining of basal cells for HSP27, was not only observed in OLPs but NOMs also showed intense staining for HSP27 and only one NOM showed mild staining. On the other hand, in their study, OLPs and NOMs showed positive equal staining for HSP27 at superior layers' keratinocytes that is to some extent in contradiction with our results because in our study, positive staining for HSP27 at superior layers' keratinocytes was not equal in OLPs and NOMs such that at suprabasal and superficial layers, NOMs showed more intense staining for HSP27 than OLPs. In their study, although HSP27 expression had been changed in OLPs, the observed difference compared to NOMs was minimal and thus insignificant and inconclusive. They concluded that expressed HSPs in OLP can help to persistence and chronicity of disease or can only be reflective of cellular damage. Their findings are to some extent in contradiction with our results because although in our study there were not significant differences between OLP and NOM groups at basal and superficial layers in the light of percentage of stained cells for HSP27, there was significant difference between OLP and NOM groups at suprabasal layer in the light of percentage of stained cells for HSP27 and percentage of stained cells was significantly higher in NOMs than OLPs at this layer. The reason for this contradiction can be related to usage of frozen samples in their study while paraffin-embedded tissue blocks were used in our study. Also, in our study OLPs included of equal number of reticular and erosive OLP but this separation of OLP subtypes was not done in their study and they might have used of OLPs with different distribution of such subtypes that we used in our study that resulted in this difference and to some extent contradictory results. Another reason could be related to division of epithelium into basal, suprabasal and superficial layers in our study but the division in this form has not been made in their study and sampled were evaluated at basal and superficial layers and keratinocyte located above the basal layer were not separated into suprabasal and superficial layers.

In Di P at al. study (8), HSP60 protein expressed much greater in OLP than other studied groups including NOM. HSP70 protein expression at epithelial cells of erosive OLP decreased compared to NOM. They concluded HSP60 and HSP70 play an important role in OLP pathogenesis.

In Seoane J et al. (9) study, no significant difference between OLP and NOM groups with respect to HSP70 expression. They concluded that HSP70 do not play a prominent role in OLP pathogenesis.

In Seoane JM et al. (4) study, none of the oral leukoplakia samples showed positive nuclear staining for HSP27 that is consistent to some extent with our results because none of the OLPs showed HSP27 nuclear staining in our study.

In Chaiyarit P study (10), significant decrease of HSP90 expression in OLPs compared to NOMs was observed. They concluded that changes in HSP90 expression might be related to pathologic stress such as chronic inflammation in OLP.

In García-García V study (5) on HSP27 expression at different clinical stages of OLP, at basal layer, overexpression of HSP27 in G2 (active atrophic OLP or with moderate activity) and G3 (inactive atrophic OLP or with mild activity) stages was observed compared to G1 (OLP with mild or moderate activity) and control group (normal mucosa). Decrease of HSP27 expression was observed at superficial layer at all stages compared to control group. They concluded that overexpression of HSP27 at basal layer, which was observed during developmental process, and lower staining at superficial layer in all OLP stages suggest that HSP27 may play a role in OLP pathogenesis. Although comparison of their results with our results is not feasible due to lack of precise homogeneity of studied groups, if we assume G2 and G3 clinical stages in their study equivalent to erosive OLP in our study and G1 clinical stage equivalent to reticular OLP, it would be possible to somewhat compare the two

studies. With this assumption, HSP27 overexpression was observed at basal layer in erosive OLP compared to reticular OLP and NOM in their study. In our study, at basal layer, there was not any significant difference between erosive OLP and NOM and also between erosive and reticular OLP with respect to percentage of stained cells for HSP27; This finding is somewhat inconsistent with their results. The reason for these inconsistencies can be related to difference of lesions' classification between our study and their study or can be related to different sample size between these two studies or can be related to difference in technical accuracy of immunohistochemical procedure between these two studies. In their study, at superficial layer, decrease of HSP27 expression was observed in all OLP stages (erosive and reticular) compared to NOM. In our study, at suprabasal and superficial layers, there was significant decrease in HSP27 expression in reticular OLP than NOM that is somewhat consistent with their results. On the other hand, in our study, at suprabasal and superficial layers, there was no significant difference between erosive OLP and NOM with respect to HSP27 expression that is somewhat inconsistent with their results. The reason for this inconsistency can be primarily related to difference of lesions' classification between our study and their study or can be related to different sample size between these two studies. They concluded that changes in HSP27 expression suggests its involvement in OLP pathogenesis that our findings also support of this conclusion.
In Slebioda Z et al. study (11) etiopathogenesis of oral aphthous disease as a chronic inflammatory disease was assessed. They stated that significant decrease in HSP27 expression was observed in oral mucosa of patients with oral aphtha

Conclusion: Our findings support HSP27 role in OLP (especially reticular subtype) pathogenesis and changes in HSP expression may be related to pathologic stress such as chronic inflammation in OLP patients.

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Conflict of interest statement: No conflict of interest

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Table legends:

Table1: Comparison of percentage of stained cells for HSP27 in basal, suprabasal and superficial layers of OLP and NOM epithelia

Percentage of stained cells / group		basal			suprabasal			superficial		
		1	2	3	1	2	3	1	2	3
Lichen planus	Number	11	1	22	7	0	27	14	4	16
	percentage	32.35	2.95	64.70	20.58	0	79.42	41.17	11.76	47.07
Normal mucosa	number	1	1	15	0	0	17	4	2	11
	percentage	5.88	5.88	88.24	0	0	100	23.52	11.76	64.72
P-value		0.1			0.04			0.43		

Table2: Comparison of staining intensity for HSP27 in basal, suprabasal and superficial layers of OLP and NOM epithelia

Staining intensity / group		Basal				Suprabasal				superficial			
		-	+	++	+++	-	+	++	+++	-	+	++	+++
Lichen planus	number	0	11	11	12	0	10	12	12	9	4	12	9
	percentage	0	32.35	32.35	35.3	0	29.42	35.29	35.29	26.47	11.76	35.3	26.47
Normal mucosa	number	0	0	10	7	0	0	10	7	0	0	10	7
	percentage	0	0	58.82	41.18	0	0	58.82	41.18	0	0	58.82	41.18
P-value		0.02				0.03				0.03			

Table3: Comparison of percentage of stained cells for HSP27 in basal, suprabasal and superficial layers of reticular and erosive OLP and NOM epithelia

Percentage of stained cells / group		basal			suprabasal			superficial		
		1	2	3	1	2	3	1	2	3
Reticular lichen planus	Number	7	1	9	7	0	10	11	2	4
	percentage	41.17	5.88	52.95	41.17	0	58.83	64.7	11.77	23.53
Normal mucosa	number	1	1	15	0	0	17	4	2	11
	percentage	5.88	5.88	88.24	0	0	100	23.52	11.76	64.72
P-value		0.04			0.003			0.03		
Erosive lichen planus	number	4	0	13	0	0	17	3	2	12
	percentage	23.52	0	76.48	0	0	100	17.64	11.77	70.59
Normal mucosa	number	1	1	15	0	0	17	4	2	11
	percentage	5.88	5.88	88.24	0	0	100	23.52	11.76	64.72
P-value		0.23			1.00			0.91		

Table4: Comparison of staining intensity for HSP27 in basal, suprabasal and superficial layers of reticular and erosive OLP and NOM epithelia

Staining intensity group		basal				suprabasal				superficial			
		-	+	++	+++	-	+	++	+++	-	+	++	+++
Reticular lichen planus	Number	0	10	5	2	0	10	5	2	6	2	8	1
	percentage	0	58.82	29.41	11.77		58.82	29.41	11.77	35.29	11.76	47.05	5.9
Normal mucosa	number	0	0	10	7	0	0	10	7	0	0	10	7
	percentage	0	0	58.82	41.18	0	0	58.82	41.18	0	0	58.82	41.18
P-value			0.001			0.001				0.005			
Erosive lichen planus	number	0	1	6	10	0	0	7	10	3	2	4	8
	percentage	0	5.88	35.29	58.83	0	0	41.17	58.83	17.66	11.76	23.53	47.05
Normal mucosa	number	0	0	10	7	0	0	10	7	0	0	10	7
	percentage	0	0	58.82	41.18	0	0	58.82	41.18	0	0	58.82	41.18
P-value			0.28			0.30				0.054			

Figure legends:

Fig.1: a) Reticular OLP with strong cytoplasmic staining in all three layers of epithelium (×400 magnification); b) Erosive OLP with strong cytoplasmic and membranous staining in all three layers of epithelium (×100 magnification); c) Reticular OLP with mild cytoplasmic staining in basal and suprabasal layers of epithelium (×400 magnification); d) Normal mucosa with moderate membranous staining in all three layers of epithelium (×400 magnification)