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Original

Phenotypic alteration of basal cells in oral lichen planus; switching keratin 19 and desmoglein 1 expression

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Abstract: In oral lichen planus, extracellular matrix and basal cells are damaged by T-lymphocytes. As a consequence, changes in expression of collagen fibers within the connective tissue and cytoskeletons of the epithelial tissue can be observed. With the goal of examining the characteristic changes undergone by basal cells as a consequence of T-lymphocytes damage in oral lichen planus, we investigated protein expression in the epithelial-connective junction. We selected 20 cases of oral lichen planus and 5 control samples of buccal mucosa. Subsequently, we divided the oral lichen planus cases into thin and thick parts based on the mean values of epithelial thickness from the control samples, and counted the positive rate of collagen IV, keratin 19, desmoglein 1, and Ki-67. Collagen IV immune-reactivity partially disappeared or thickened in oral lichen planus. The keratin 19 positive rate in oral lichen planus cases was significantly lower than in the controls. Desmoglein 1 positive rate of the thick part was significantly higher compared to the thin part of oral lichen planus. Thus, modifications in basal cells with both reduced keratin 19 expression and alterations of desmoglein 1 expression suggest that in oral lichen planus, as a consequence of cell injury or regeneration in the interface area, there is a disappearance of the "true basal cell nature".

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Introduction

Oral lichen planus (OLP) is a chronic inflammatory disease that occurs in the epithelial-connective tissue interface due to damage caused by T-lymphocytes (1). Although its etiology is to date unclear, various conditions such as genetic disorder, stress, drugs, dental materials, or hepatitis C virus seem to be linked to the disease (2,3). The clinical and histopathological features characterize the entity of this disease (4).

CD8-positive T-lymphocytes activated by unknown antigens infiltrate in a band-like fashion below the epithelium. As a result, basal cells undergo degeneration due to liquefaction and apoptosis induced by Fas ligand or TNFα (5). These injuries are characteristic in the interface between epithelium and connective tissue of OLP. In the epithelium-connective tissue interface, the basement membrane ruptures with partial effacement of basement membrane proteins (6). Additionally, collagen type 4 (COL4), a main component of these proteins, is degraded by metalloprotease 9 produced in the course of inflammatory reactions (7-9).

As the interface undergoes degenerative changes, the oral epithelium demonstrates variable abnormal features, such as hyperkeratosis or abnormal keratinization of the spinous layer, including acanthosis (10,11). Immunohistochemically, non-keratinized epithelial markers, such as keratin 13 (K13), disappear in OLP. Instead, intermediate filaments of keratinized squamous epithelium, keratin 1 (K1) or keratin 10 (K10), are abnormally expressed and are characterized by an increased mRNA expression (11,12). While normal oral basal cell epithelium expresses

Table 1 Summary of primary antibodies

Antibody	Clone	Company	Dilution	Pretreatment
COL4	CIV22	Dako	1:50	ProK
K19	b170	Leica	1:100	ProK
DSG1	Dsg1-P23	PROGEN	Ready to use	HRM
Ki-67	MM1	Leica	1:100	HRM

COL4: Collagen type IV, K19: Keratin 19, DSG1: Desmoglein 1.
ProK: Protenase K, HRM: Heat antigen Retrieval Method.

keratin 19 (K19), the latter either disappears from basal cells in both hyperplastic lesions and OLP (11,13), or it becomes aberrantly expressed in supra-basal or spinous cells of dysplastic oral epithelium (14).

Keratinocytes differentiate toward superficial layers of the oral stratified squamous epithelium. Desmosome contains desmosomal proteins, such as desmoglein (DSG) and desmocollin, and it has an important role in the epithelial differentiation (15). DSG1 is linked to cell proliferation and has a key role in squamous cell differentiation. In proliferative epithelium such as basal cells there is a transition of DSG1 reactivity from negative to positive (16).

As mentioned above, during both cell damage and cell proliferation, basal cells of the oral squamous epithelium undergo changes. Therefore, in the present study, we focus on OLP' basal cells phenotypic changes in absence of significant atypical features. Specifically, we evaluate the expression of K19 and DSG1 basal cell markers in comparison with the expression of COL4 and Ki-67 markers for cell damage and proliferation.

Materials and Methods

Case selection

From the archives of our surgical pathology laboratory, we selected OLP 130 cases diagnosed based on a set of modified WHO diagnostic criteria (4). Specifically, these comprised clinically of bilateral and lace-like network features, and histologically of a band-like lymphocytic infiltration and liquefaction degeneration in the basal cell layer in absence of epithelial dysplasia. Furthermore, we chose 20 additional cases, which were bilateral and reticular with no history of either drug and metal induced allergy or hepatitis C virus infection. All OLP specimens demonstrated typical histopathological features, including: band-like infiltration of lymphocytes, variable degrees of basal damage, effacement of basal cell alignment, saw tooth appearance of rate ledge, and abnormal keratinization. Five specimens of buccal mucosa where chosen as controls due to the absence of inflammation and atypical changes. These control specimens showed absence or minimal keratinization. The average ages of controls and experimental OLP group were 49.8 and 60.7 years, respectively.

Morphometrical analysis

All specimens excised were fixed with 10% neutral buffered formalin and embedded in paraffin after routine processing. Hematoxylin and eosin stained sections were processed by × 1,024 pixels digital imaging which measured 433 μm in width. Images were captured using a microscope imaging system (BX51, Olympus, Tokyo, Japan). Using an image analysis software (WinRoof, Fukui, Japan), five portions of the epithelial full thickness of control samples were randomly measured in the above-mentioned imaging process and mean values for the controls were calculated. In OLP, two parts of full thickness, the thinnest and thickest parts, were measured. The mean values of the epithelial full thickness were 262.2 and 268.7 μm in the control and OLP, respectively. Based on the mean value of the control's thickness, 40 thickness portions of OLP were divided into the thin and thick parts based on their values being lower and higher than 262.2 μm, respectively. Eleven of 20 cases included both the thin and thick parts. These 11 cases were used for paired test. Thickness values of keratinized and spinous layers were analyzed using the same criteria of the full thickness values.

Semi-quantitative analysis of basal cell damage

Basal cell damage was divided into four grades as follows: Grade 0 (G0), no damage; Grade 1 (G1), separation between basal cells; Grade 2 (G2), infrequent cytoplasmic vacuolization of basal cells; and Grade 3 (G3), liquefaction or hydropic degeneration of basal cells. Grade was evaluated in both the thinnest and thickest parts of OLP.

Immunohistochemistry

Deparaffinized and hydrated 3 micron-thick sections were pretreated according to the commercial recommendations. A high-temperature unmasking technique was performed by the autoclave at 121°C for 15 min in 0.01 M sodium citrate buffer solution (pH 6.0), and some

sections were digested with proteinase K (DAKO, Glostrup, Denmark). As shown in Table 1, COL4 (DAKO, Glostrup, Denmark), K19 (Leica Biosystems, Newcastle, UK), DSG1 (PROGEN, Heidelberg, Germany) and Ki-67 (Leica Biosystems, Newcastle, UK) were used as primary antibodies. All antibodies were incubated at 4 °C for 24 h. Nichirei MAX-PO Multi (Nichirei, Tokyo, Japan) was used as secondary antibody. Sections were incubated at room temperature using HISTOSTAINER (Nichirei) for 30 min. After visualization with 3-3'-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark), sections were counterstained with hematoxylin. Negative control slides were processed with phosphate buffered saline instead of primary antibodies.

Quantitative analysis of immunoreactivity

The positive representation of each antibody was analyzed as follows: the COL4 positive representation was given by the value of the number of basal cells located in the COL4 positive areas divided by the number of all basal cells in the examined areas. Both K19 and DSG1 positive representations were given by the number of positive basal cells divided by the number of all basal cells examined. Ki-67 positive representation was based on the number of positive cells divided by all cells comprising the lower three layers examined.

Statistical analysis

We used Fisher's exact test to compare the number of antibodies positive cases between controls and OLP. Prior to comparison analysis, normality and homogeneity of variances were confirmed by the Shapiro-Wilk normality test and the Bartlett test, respectively. For the non-parametric statistical hypothesis tests, Mann-Whitney *U* test and Wilcoxon rank test were used. Specifically, these tests compared values of controls versus OLP and values of thin parts versus thick parts as repeated measurements on a single sample, respectively. Student *t*-test was used to analyze Ki-67 indices.

Multiple comparison analysis between internal-control and different-phenotypic groups was performed using Kruskal-Wallis test. Values less than 5% were considered statistically significant. Statistical analysis was performed using the free software "R" (The Foundation for Statistical Computing, version 2.13.0) (17).

Ethics

This study was approved by the Ethics Committee of Matsumoto Dental University (Approval Number 209) and conducted according to Helsinki Declaration principles (version 2002).

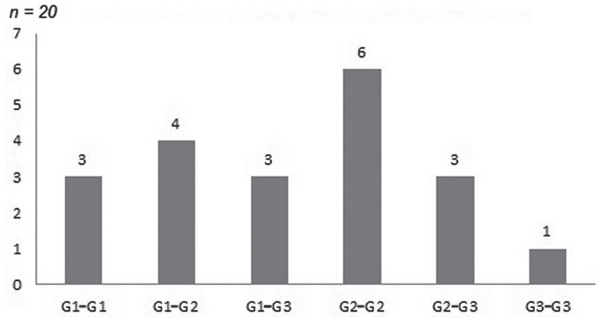

Fig. 1 Grade distribution of basal cell damage in pairs of thick and thin portions of OLP cases.

Results

The mean values of keratinized layer thickness were 15.4 and 45.3 μm in the control and OLP, respectively. They both reached statistical significance ($P < 0.001$). The values of thick and thin parts were 46.6 and 37.6 μm, respectively. The value of the thick part was significantly higher ($P < 0.001$) than that of the thin part. The mean values of spinous layer thickness of OLP and controls were 175.7 and 129.5 μm, respectively. However, these values did not reach statistical significance. On the contrary the values of thick and thin parts were 316.7 and 127.0 μm, respectively. The value of the thick part was significantly higher ($P < 0.001$) compared to the thin part.

While the controls show no significant changes in basal cells, variable degrees of cell damage were observed in OLP. In the thick and thin parts evaluated, combinations of G1-G1, G1-G2, G1-G3, G2-G2, G2-G3, and G3-G3 were found in 3 (15%), 4 (20%), 3 (20%), 6 (30%), 3 (15%), and 1 (5%) of 20 cases, respectively (Fig .1). The mean value of cell damage in the thin part was significantly ($P < 0.05$) lower compared to the thick part (G3 vs. G2).

COL4 was constantly positive in controls, while it partially disappeared in OLP, accompanied by infrequently thickening of COL4 positive basement membrane (Fig. 2a). COL4 positive rate in OLP was significantly lower compared to the controls (100 vs. 77.8%, $P < 0.001$). However, no statistically significant difference between values of the thin and thick parts was observed (Table 2).

K19 was positive in most basal cells of the controls but partially negative in basal cells of OLP (Fig. 2b). K19 positive rate of OLP was significantly lower compared to the controls (83.1 vs. 0.0%, $P < 0.001$). While K19 had decreased expression in the thick part compared to the thin part, a comparison of K19 expression between the thin and thick parts showed no significant difference

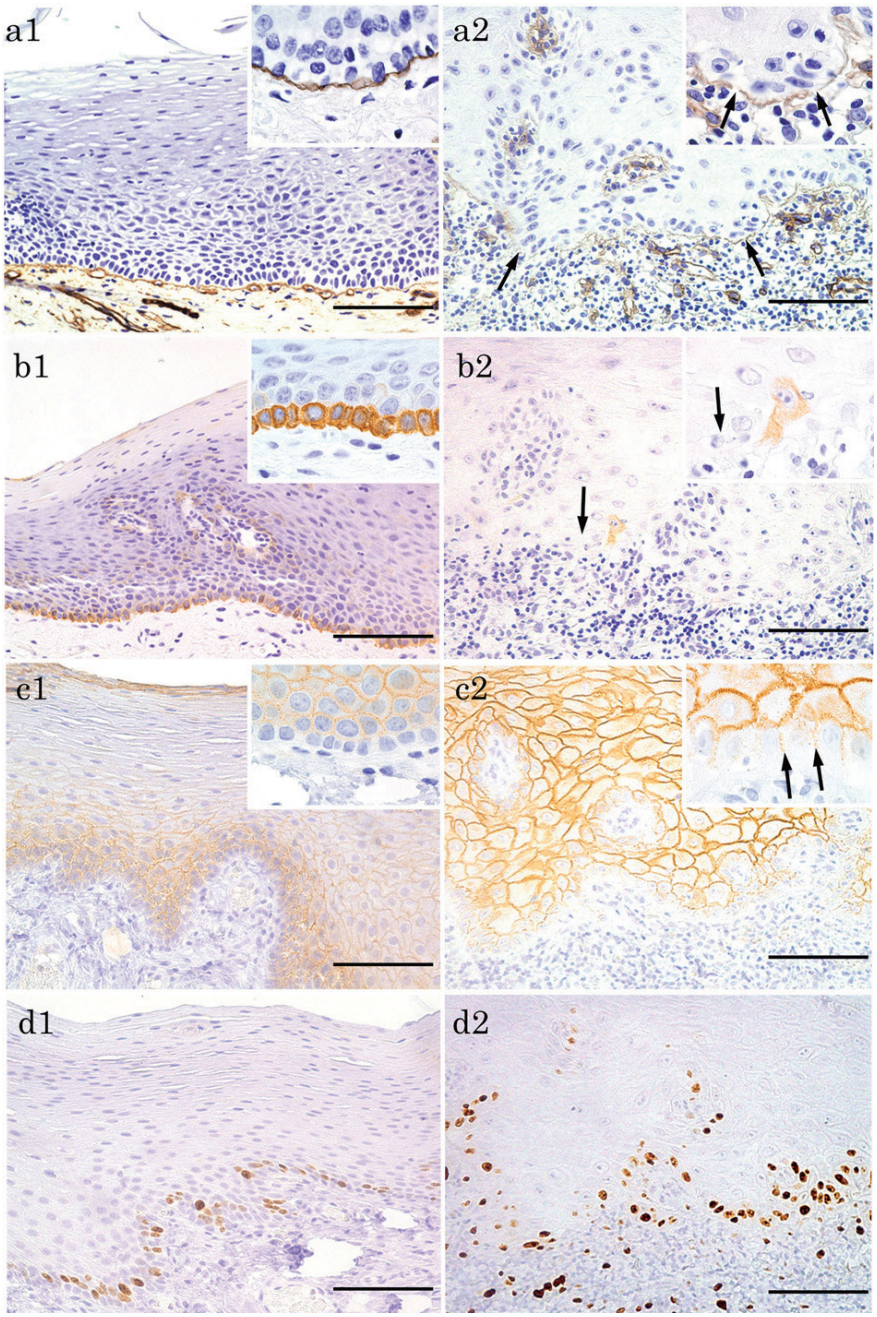

Fig. 2 Immunohistochemical features of the epithelial-connective tissue interface.
a COL4 immunostaining shows continuous positive reaction in the control (a1) but various degrees of negative reaction (arrows) in OLP (a2).
b K19 is constantly positive in the control's basal cells (b1) but mostly negative (arrows) in OLP (b2).
c DSG1 is mostly negative in basal cells of the control (c1) but partly positive (arrows) in the thick part of OLP (c2).
d Ki-67-positive cells are found in the parabasal layer of the control (d1), while those cells are randomly scattered in lower three layers of OLP (d2). All bars indicate 100 μm.

(Table 2). Comparing cell damage values between the controls and each group showed significant differences between the control values and values of G1 ($P < 0.001$), G2 and G3 ($P < 0.01$, Fig. 3). However, there was no statistical significance among values of G1, G2, and G3.

DSG1 was almost negative in basal cells of the controls and OLP. However, basal cells of OLP partially showed a DSG1 positive reaction (Fig. 2c). While no significant difference among DSG1 positive rates in the controls versus the OLP was observed, DSG1 positive rate of the thick part was significantly higher compared to the thin part (41.7 vs. 0.0%, Table 2, $P < 0.05$).

Table 2 Positive rates of antibodies against epithelial-connective tissue junction

	Cont	OLP	Statistics
COL4	100	77.8	$P < 0.001$
K19	83.1	0	$P < 0.001$
DSG1	7.3	9.1	
Ki-67	42.5§	38.5§	

	Thick	Thin	Statistics
COL4	87.1	72.9	
K19	0	5.3	
DSG1	41.7	0	$P < 0.05$
Ki-67	40.6§	45.4§	

Values are median percentage and values with"§" are mean percentage.

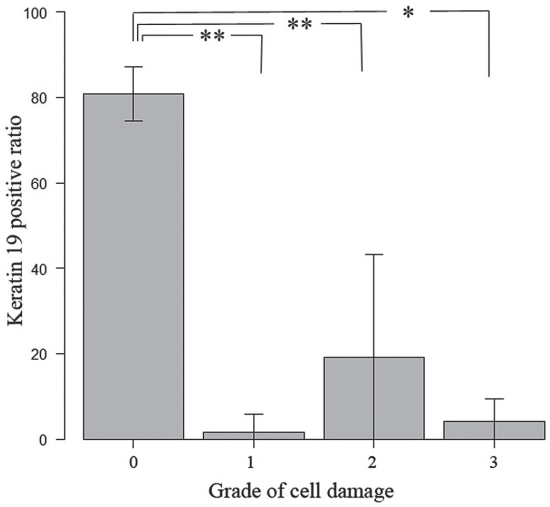

Fig. 3 Multiple comparisons of K19 positive ratios among grades of basal cell damage. Values of grade 1 to 3 (OLP) are significantly lower than those of grade 0 (control).

Ki-67 positive cells were mostly seen in the parabasal layer of the controls. Interestingly, these cells were scattered throughout the lower three layers of OLP (Fig. 2d). The Ki-67 positive rate of the controls was similar to that of OLP (42.5 vs. 38.5%) and there was no significant difference between values of the thin and thick parts (Table 2).

Discussion

Semi-quantitative analysis reveals that basal cell damage of the thin part is significantly more severe than that of the thick part. These results are consistent with the epithelial thinning phenomenon, which is due to degeneration or apoptosis (5). Decrease in COL4 positive ratio also demonstrates that basal cells are losing the support of the basement membrane. This condition represents the destruction of the epithelial-connective tissue interface in OLP. Of note, the COL4 positive ratio among the thin and the thick parts is not significantly different, even though the thin part's cell damage is more severe compared to the thick part. As shown in a previous study (6), the basement membrane demonstrates partial effacement and thickening. It appears that complex changes consisting in degenerative and regenerative reactions occur along the interface between basal cells and connective tissue. Therefore, COL4 loss is not proportional with the severity of damage.

Injured OLP epithelium has a decreased expression of K19 with significant disparity in positive values between controls and OLP. K19 is a basal cell marker of non-keratinizing squamous epithelium (18). A number of possibilities can explain K19 disappearance in this case. One possible cause can be the removal of basal cells due to apoptosis or degeneration, which leaves the parabasal or spinous cells exposed against the connective tissue. Additionally the phenotypic changes of basal cells could occur as a consequence of basal cell injury or damage of the extracellular matrix and basement membrane in OLP (19,20). The expression pattern of K19 appears to be altered following both inflammatory changes and hyperplastic epithelium (14). Some investigators reported an increased proliferation rate of the basal cells of OLP (21). Prior to this investigation, we hypothesized that cell proliferation may influence the immunophenotype of basal cells. Unexpectedly, in the present study, we did not observe a disparity in Ki-67 expression between the controls and OLP. Furthermore, no correlation between K19 and Ki-67 expression levels were found in this study. Interestingly, we observed that expression levels of these markers in the epithelial full thickness of the controls and OLP are not significantly different. Although K19 reactivity shows a tendency to decrease in thick parts versus thin parts, there is no significant difference between thin and thick parts. Based on these results, we believe that a reduced K19 immunoreactivity is one of the characteristic pathological changes of OLP. Additionally, there is a significant difference in K19 expression between the controls and OLP at all grades of cell damage. We believe that these results may mean that K19-immunophetype of basal cells is altered due to degeneration rather than proliferation of basal cells.

OLP epithelium in thick parts demonstrates a higher thickness value of the keratinizing layer compared to the thin parts. While values of spinous layer thickness

do not reach a significant difference statistically when comparing controls and OLP, it seems that hyperkeratosis develops mostly in the thick epithelium of OLP rather than in injured-thin epithelium. When comparing cell proliferation indices between the controls and OLP, no statistically significant difference is observed. However, Ki-67 positive cells' distribution appears to be irregular in the lower three layers of OLP. In cutaneous lichen planus, the epithelial turnover is prolonged as a consequence of basal cell damage caused by lymphocytes infiltrating beneath the epidermis. Epidermal cells require more time for terminal differentiation (22). Although the epithelium of OLP is not characterized by an increase in cell proliferation activities, regenerative changes might slowly take place in the epithelium.

DSG is a major structural protein contained in a desmosome that has an important role in both epithelial proliferation and differentiation (15). DSG1 bound with Erbin, also known as an ERK regulator, suppresses the EGFR/ErbB1 signaling pathway by inhibiting the formation of Ras/Raf complex and accelerates the differentiation of keratinocytes above the basal layer (23,24). Furthermore, in vitro shRNA depletion of DSG1 results in the reduction of K1/K10 expression in cultured monolayers of normal human epidermal keratinocytes and causes the development of abnormal epidermal architecture in skin models (25). In the potentially malignant disorder of oral epithelium, proliferating neoplastic basal cells also show DSG1 negative immunoreaction which represents basal cell nature (26). While basal cells of normal oral epithelium lack DSG1 expression, in OLP, some basal cells are positive in absence of a statistical significance. Interestingly, detailed observation of DSG1 expression shows a significant difference between thin and thick parts in OLP. This finding suggests that DSG1 expression in basal cells of thickened-OLP epithelium leads to a repression of proliferation activity, which is in line with the above-mentioned results of reduced Ki-67 expression. Again, we emphasize that the epithelium of the thick part is characterized by abnormal hyperkeratosis. Taken together, these results support the hypothesis that abnormal keratinization and DSG1 expression of basal cells have an intimate relationship with each other.

In conclusion, the epithelium of OLP shows an abnormal phenotypic alteration of basal cells characterized by reduced K19 and increased DSG1 expression. These findings are thought to be a representative feature in the disappearance of the "true basal cell nature" after cell injury or regeneration in the interface area of OLP. These changes are characteristic of OLP.

Conflict of Interest

There is no potential conflict of interest.

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