Original

Loss of Basal Cell Character in Regenerating Oral Squamous Epithelium with Altered Expression of Desmoglein 1, Desmocollin 3 and Keratin 19

Hirokuni Ko1,2, Hiromasa Hasegawa3,4, Takanaga Ochiai2,3,4, Katsumitsu Shimada4,6, Rita Rani Roy3, Sohichi Aizawa4 and Haruki Yamada4,6

1 Department of Oral Pathology, Matsumoto Dental University, Shiojiri, Japan
2 Kou Dental Clinic, Osaka, Japan
3 Graduated School of Oral Medicine, Matsumoto Dental University, Shiojiri, Japan
4 Surgical Pathology Unit of Matsumoto Dental University Hospital, Shiojiri, Japan
5 Department of Oral Surgery, Yokohama Chuo Hospital, Yokohama, Japan
6 Surgery Unit, Iwaki Kusakidai General Clinic, Iwaki, Japan

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Abstract: To clarify the phenotypic changes of basal cells of oral squamous epithelium during pathological regeneration, 40 cases diagnosed as mucous cyst and 10 cases including normal epithelium were used as experimental and control subjects. Antibodies against desmoglein 1 (DSG1), desmocollin 3 (DSC3), keratin 19 (K19) and Ki-67 nuclear antigen (Ki-67) were used for immunohistochemical examinations. Ki-67 index values and the combinations of DSG1, DSC3 and K19 reactivity were analyzed by using hierarchical clustering and one-way ANOVA. In contrast to the controls, experimental specimens showed DSG1 positive, DSC3 negative and K19 negative, in 50%, 10% and 87.5% of the experiments, respectively. Cluster hierarchy analysis divided all samples including controls and experiments into three clusters mostly demonstrating DSG1+/DSC3-/K19-, DSG1-/DSC3+/K19+ and DSG1-/DSC3+/K19- as clusters 1, 2 and 3, respectively. Cluster 1 showed significantly higher values of Ki-67 than did cluster 3. The value of DSG1 positive was statistically significant compared with that of DSG1 negative. Our results show that the alterations from DSG1 negative to DSG1 positive could be crucial for cell proliferation, accompanied by K19 loss, which is consistent with the effects of regulatory molecules such as SIRT2 or EphA2 following EGFR-upregulation. We have to be aware that the loss of basal cell character with DSG1 and K19 alterations are basic phenomena in pathological regeneration, and abnormal DSC3-loss infrequently occurs in non-neoplastic conditions.

Key words: Desmocollin 3, Desmoglein 1, Keratin 19, Oral squamous epithelium, Regeneration

Introduction

Oral squamous epithelium shows well-organized stratification that is composed of basal, spinous, and parakeratotic surface cells. These cells express different phenotypes such as desmosomal protein or keratins. Desmosomes are common in epithelial tissues where they anchor keratin intermediate filaments to the membrane. Desmoglein (DSG) 1 and desmocollin (DSC) 3 are major transmembrane glycoproteins of Ca2+-dependent molecules that anchor adjacent epithelial cells to one another. There are four isotypes of DSG and three isotypes of DSC. Variations in these molecular desmosomal proteins and keratin distributions exist in the desmosomes of different cell types. Simple epithelium expresses only DSG2 and DSC2, whereas stratified squamous epithelium has all isotypes of DSG and DSC in different patterns. Stratified squamous epithelium primarily expresses pairs of DSC1/3 and DSG1/3 but it lacks expression of DSC1 and DSG1 in the lateral junctions of basal cells, which can be a characteristic nature of basal cells. Keratins are intermediate filaments that are also well known as makers for epithelial typing or differentiation. Of these intermediate filaments, keratin 19 (K19), which is a type I keratin, is expressed not only in most simple epithelia, notably in various ductal epithelia, in small and large intestinal epithelium, in gastric favela epithelium, and in mesothelium, as well as in basal cells of non-keratinizing stratified squamous epithelium. This keratin is believed to be a possible specific marker for epidermal stem cells.

Numerous studies have shown altered expressions of desmosomal proteins and keratins in dysplastic and malignant changes of squamous epithelium. Reduced expression or loss of DSG1 and K19 were noted in oral borderline line malignancies or squamous cell carcinomas (SCCs). DSC3 is also available as a progression marker of skin tumors and a marker for distinction between pulmonary adenocarcinomas and SCCs. As described above, desmosomal proteins and keratins are widely applied in surgical pathology use. However, desmosomal proteins and keratins are altered not only in neoplastic lesions but also in inflammation such as oral lichen planus. It is controversial whether abnormal expressions, namely loss or aberrant expression of these proteins, are reminiscence of potential change towards malignancy. We have limited data of desmosomal proteins and keratins in non-neoplastic proliferation phenomena of oral squamous epithelia.

Therefore, we aimed to clarify the relationship between cell kinetics and phenotypes, focusing especially on the expression of DSG1 and...
characterizing basal cells in the regenerative epithelium of oral mucosa.

**Materials and methods**

**Sample selection**

In our study, we used the overlying epithelium of mucoceles as a model of the pathological regeneration of oral squamous epithelium because oral mucoceles microscopically show attenuated surface epithelium and possible regeneration of the epithelium across the floor of the blister. Then, we selected 40 archived cases of mucoceles that demonstrated various degrees of regeneration of overlying epithelium. All cases were diagnosed as mucoceles at the Surgical Pathology Unit of Matsumoto Dental University Hospital. Twenty cases occurred in the lower lip and 20 cases arose from the anterior tongue after histological confirmation that all lesions were covered by squamous epithelium with regenerative changes such as basal cell hyperplasia, drop-shaped proliferation or marked epithelial thickening. To avoid influences of tissue preparation such as fixation for immunohistochemistry, ten out of 40 cases containing epithelium with a normal immunophenotype adjacent to cystic lesions were used as internal controls. Mean age of selected cases was 18.6 (ranging from 4 to 68 years old) and the male to female ratio was 1: 2.3.

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**Table 1. Panel of primary antibodies**

| Monoclonal antibody | Source | Clone | Treatment | Dilution |
|---------------------|--------|-------|-----------|----------|
| anti-desmoglein 1   | (DSG1) | Dsg1-P23 | HIE, pH6 | Ready to use |
| anti-desmocollin 3  | (DSC3) | Dsc3-U114 | HIE, pH6 | 1:10 |
| anti-cytokeratin 19 | (K19)  | b170 | Digestion | 1:50 |
| anti-human Ki67     | (Ki67) | MM1 | HIE, pH6 | 1:200 |

HIE: heat induced epitope retrieval
All specimens excised were fixed with 10% neutral buffered formalin and embedded in paraffin after routine processing. Deparaffinized and hydrated 3 micron-thick sections were pretreated according to commercial recommendations. High-temperature unmasking technique was performed by the autoclave at 121°C for 15 minutes in 0.01 M sodium citrate buffer solution (pH6.0), and some sections were digested with proteinase K (DAKO, Glostrup, Denmark). As shown in Table 1, antibodies to desmoglein 1 (DSG1), desmocollin 3 (DSC3), keratin 19 (K19) and Ki-67 nuclear antigen (Ki-67) as primary antibodies and Nichirei MAX-PO Multi (Nichirei, Tokyo, Japan) were incubated at room temperature using HISTOSTAINER® (Nichirei, Tokyo, Japan) for 60 minutes and 30 minutes, respectively. After visualization with 3-3'-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark), sections were counterstained with hematoxylin. Negative control slides were processed without the primary antibodies.

**Evaluation of immunohistochemistry**

Desmosomal proteins (DSG1 and DSC3) and K19 expression in basal cells were assessed as follows. Cases with positive cells less than 50% and equal or more than 50% in the basal layer were evaluated as negative and positive, respectively. Ki-67-labelling index (Ki-67 index) was evaluated under medium power fields using image analysis software WinRoof® (Mitani Corp., Fukui, Japan). Evaluated cells were over 500 in number, with mean values of 1018.4 and 761.4 cells in control and experimental cases, respectively.

**Statistical analysis**

Prior to analyses, normality, equality of two variances and equality of multiple variances were confirmed by Shapiro-Wilk normality test, F-test and Bartlett test, respectively. The relationship between age and Ki-67 index was checked by Pearson product-moment correlation. To classify cases phenotypically, cluster hierarchy analysis was performed using Ward’s method. Comparison of Ki-67 indices between controls and experiments and phenotypic groups were analyzed using T-test and one-way ANOVA followed by a Tukey test as a post-hoc analysis. All were analyzed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan)17), which is a graphical user interface for R (www.r-project.org) (The R Foundation for Statistical Computing, Vienna, Austria). Values less than 5% were considered statistically significant.

**Ethics**

This study was approved by the Ethics Committee of Matsumoto Dental University (#127) and conducted according to Helsinki Declaration principles (version 2002). Informed consent was obtained in the form of opt-out because all subjects were archived paraffin-embedded tissues. Prior to examination, the information for each subject was anonymized for personal privacy protection.

**Results**

**Immunohistochemical staining profiles**

Ten control samples showed the following same findings. DSG1 was positive in parabasal to surface cells but linearly negative in basal cells. DSC3 was positive in the lower three layers, namely in basal, parabasal and spinous layers but negative in the surface. K19 showed a linear-positive reaction in the basal layer (Fig. 1a-1d).

Twenty cases (50%) of regenerative epithelium showed DSG1-negative basal cells (Fig. 3a and 4a) but the other half of cases had DSG1-positive basal cells (Fig. 2a). DSG1 negative cells were sometimes piled up from basal to parabasal layers (Fig. 4a).

DSC3 was mostly demonstrated in basal to parabasal layers except for the surface (Fig. 2b and 3b). However, four cases (10%) showed that DSC3-negative cells aligned in the basal and parabasal layers (Fig. 4b).

K19 was mostly effaced in the basal layer of 35 (87.5%) experimental cases. 30 cases (76.9%) showed complete effacement (Fig. 2c) and the remaining 5 cases (12.8%) showed partial loss (Fig. 3c and 4c). Consequently, 38 cases (95%) showed decreased expression of K19 in the basal layer. There were only two cases (5%) showed a normal looking feature, namely fully K19-positive basal cells. Six cases (15%) demonstrated a suprabasal aberrant positive reaction in the spinous layer from slight to marked hyperplastic change (Fig. 3c).

In experimental cases, Ki-67 positive cells were mostly observed in the parabasal layer (Fig.1d). In experimental cases, 27 of 40 (68%) cases showed that Ki-67 positive cells were observed in not only the parabasal layer but also in the basal layer and irregularly aligned in double or multiple-layered features (Fig. 2d, 3d and 4d). There were some markedly hyperplastic or acanthotic cases, with or without K19, that did not...
demonstrate any Ki-67 positive cells (Fig. 3d and 4d).

**Immunophenotypes of basal cells**

Consequently, experimental cases showed six patterns of combinations of DSG1, DSC3 and K19. A normal immunophenotype (DSG1-/DSC3+/K19+) was seen in only one case, and the other 39 cases showed abnormal immunostaining patterns that were DSG1+/DSC3+/K19-, DSG1+/DSC3+/K19-, DSG1-/DSC3+/K19-, DSG1-/DSC3+/K19+, DSG1+/DSC3+/K19+ were 19, 15, 3, 1 and 1, respectively (Fig. 5).

**Cluster hierarchy analysis**

All 50 samples, including control areas, were divided into three major clusters designated as Cluster 1, 2 and 3 that consisted of 19, 15 and 16 cases, respectively, by immunostaining patterns (Fig. 6). Cluster 1 showed DSG1+/DSC3+/K19-. Cluster 2 was almost normal phenotype showing DSG1-/DSC3+/K19+ but it included two exceptional cases; one “DSG1+/”DSC3+/K19+ case and one DSG1-”DSC3-”/K19+ case. All controls were sorted into this cluster. Cluster 3 was mostly DSG1-/DSC3+/K19-, but three exceptional cases of DSC3- were seen.

**Statistical analysis**

The mean value 18.0% of experimental Ki-67 index was lower than the 18.9% of the controls’ index but was not statistically significant. The correlation coefficient between age and Ki-67 index of experiments was -0.2826, showing a poor correlation. Multiple comparison tests using one-way ANOVA only revealed a statistically significant difference of mean values of Ki-67 indices between DSG1 negative and positive cases representing 13.9% versus 22.2%, respectively, with p<0.01. However, there were no significant differences between values of other phenotypes (Fig. 7). According to recalculation after clustering analysis, Ki-67 indices decreased from cluster 1 to cluster 3 as 22.3%, 19.8% and 11.8%, respectively. Statistically, there were significant differences of Ki-67 indices between cluster 1 and cluster 3 and between cluster 2 and cluster 3 with p<0.001 and p<0.05, respectively (Fig. 8).

**Discussion**

Many types of desmosomal cadherins and keratins are available for surgical diagnosis as markers of differentiation or progression markers of tumor cells. However, we have not fully possessed knowledge of these markers even in normal tissues or physiological conditions. Therefore, the purpose of this investigation has been to clarify relationships between cell proliferation and basic markers of basal cells of oral epithelium in regenerative condition, that is, non-neoplastic condition.

Consequently, we clearly demonstrated that phenotypic change of DSG1 and K19 occurred during cell proliferation in the regenerative oral stratified squamous epithelium. It seems that phenotypic change of oral epithelial tissue could be crucial for proliferation of keratinocytes, especially on DSG1 and K19. In addition, we should pay attention to these phenotypic changes when we perform immunohistochemistry in neoplastic lesions of the oral mucosa.

Normal phenotype of basal cell

All control specimens in this study showed DSG1-/DSC3+/K19+. In oral squamous epithelium, basal cells lack DSG1 immuno-reaction except for at the interface between basal and suprabasal keratinocytes. This cadherin is first expressed in the epidermis as keratinocytes transit from the basal layer[9], whereas DSC3, but not DSC1, it also present in desmosomes of the basal as well as suprabasal cell layers of mucosal stratified epithelia, including oral mucosa[9]. In an immunofluorescence staining analysis, the intensity of DSC3 expression fades gradually in the suprabasal layers and completely disappears below the upper limit of desmosomes, even though mRNA is expressed in full thickness of the epithelium[10]. Including K19 positive pattern, that is a phenotypic characteristic of keratinocytic stem cell[6,7], the normal basal cell phenotype is DSG1-/DSC3+/K19+, which is consistent with the result of our control specimens. The experimental cases classified into cluster 2 by clustering analysis were also of the normal phenotype, which might represent status post regeneration.

Lower tendency of proliferation activity in regenerative epithelium

Although the Ki-67 index of controls had a tendency to be higher than that of all experiments, control specimens constantly showed positive reaction in parabasal layer. As is well known, oral squamous epithelium physiologically renews itself within 6 days according to investigations using rats[21]. Appearance of parabasal Ki-67 positive cells is consistent with physiological phenomenon of constantly proliferating keratinocytes. On the other hand, experimental cases in the present study demonstrated irregularly aligned positive cells within several layers from the bottom, accompanied by areas effaced Ki-67 positive cells. Experimental specimens sometimes showed the intermingled areas of increased and decreased numbers of Ki-67 positive cells, which consequently resulted in a lower Ki-67 index value comparison with that of controls (18.0 versus 18.9).

Phenotype of regenerating keratinocyte

As described in the results, clustering analysis divided all experiments into three clusters (Fig. 6). However, majority of experiments were classified into K19 negative clusters, designated as clusters 1 and 3, which could be interpreted to mean that the loss of K19 seems to be essential for epithelial regeneration in pathological conditions.

The difference between clusters 1 and 3 with significantly different Ki-67 indices was the immunoreactivity of DSG1positive and negative, respectively. Because basal cells are characterized by both DSG1[9] and K19[9], the alteration of these markers could intimately relate with cell proliferation. Cluster 1 showing abnormal phenotype of K19-/DSG1+ represents complete loss of basal cell character. In addition, Ki-67 index of cluster 1 showed a higher tendency than that of cluster 2, the normal phenotype, and significantly higher than that of cluster 3. Therefore, the alteration to K19-/DSG1+ is considered as crucial for cell renewal or cell proliferation.

Sirtuin-2 (SIRT2), a NAD+-dependent deacetylase, inhibits the expression of K19, while it stimulates the expression of loricrin, a major protein component of the cornified cell envelope[22,23]. SIRT2 is regulated by ERK1/2 by increasing the protein levels[24]. On the other hand, DSG1 is up-regulated by activation of EphA2, a receptor tyrosine kinase, through ephrin-A1 ligating. In an in vitro study, the treatment of ephrin-A1 resulted in the expression not only DSG1 but also keratin 10, a marker of terminal keratinocyte differentiation. EphA2 levels were increased when the EGFR-ERK1/2 signaling pathway was activated[22,24]. In those ways, both K19-loss and DSG expression are regulated through cell proliferation signaling, which seems to give an appropriate explanation for our results.

Aberrant K19 expression

In the current study, we found suprabasal aberrant positive reaction of K19 in six cases (15%) showing slight to marked hyperplastic change. The aberrant K19 positive foci showed a tendency of lower Ki-67 positive values, as presented in Fig. 3d. Aberrant expression of K19 was frequently observed not only in oral epithelial dysplasia (OED) but also in well and poorly differentiated SCCs[22,24]. Although K19 was generally downregulated, it was considerably retained in many cases of OED or SCC[11]. These diverse expression patterns could reflect histologic differentiation, but it seems to be neither a sensitive nor a specific marker of premalignancy or malignancy. Our result demonstrated that K19 aberrant expression in suprabasal keratinocytes could occur even in non-neoplastic proliferation, probably in over-proliferation.

Chen et al[29] examined the relationships between proliferation activities and K19 expression in several epidermal tumor cells such as actinic keratosis, Bowen disease and SCC. Interestingly, they reported that the majority of K19 positive cells were Ki-67 negative. Their result is consistent with ours presented in Fig. 3d, which represents a lacking proliferative activity of hyperplastic keratinocytes.

As described above, the K19-loss is regulated by SIRT2, while the K19 expression of non-neoplastic keratinocytes is poorly understood. In squamous cell carcinoma cell lines, K19 expression could be regulated by Krüppel-like factor (KLF) 4 and Sp1, which are zinc-finger transcription factors and involved in cellular control of cell-cycle regulation and differentiation[30]. Although KLF4 has a complex function in cell cycle, the proportion of Ki67-positive cells increased in KLF4 knockout mice[31]. Together with both previous findings and our results, K19 aberrant expression seems to suppress over cell growth in regenerative foci through these transcription factors in order to maintain homeostasis.

Loss of DCS3 expressions

While DSG1 and K19 were dramatically altered in our cases, DSC3 was mostly positive. There were only 4 cases showing DSC3 negative (Fig. 4c). The phenotypes of these 4 cases were DSG1-/DSC3-/K19- and DSG1-/DSC3-/K19+, accounting for 1 case of cluster 2 and 3 cases of cluster 3, respectively. The DSC3-loss seems to be an interesting change because this cadherin is well preserved in squamous epithelium even in oral carcinoma in situ[32]. Some authors emphasize that DSC3 is a sensitive and specific marker for the diagnosis of adenocarcinoma and SCC[13] or the loss of DSC3 expression concomitant with tumor cell dedifferentiation and tumor progression[12,13]. In normal cells, DSC3 affects keratinocyte differentiation through beta-catenin stabilization and signaling[33]. Judged together with these data, DSC3 negative status might show an immature character of keratinocytes.

Interestingly, all DSC3 negative cases were classified into cluster 2 and cluster 3 but not into cluster 1 (Fig. 6), which seems to suggest that DSC3 negative cases represent suppressed proliferation activity. From this viewpoint, DSC3 negative basal cells with DSG1-/K19 phenotype seem to make themselves stay more immature than normal basal cells in order to avoid excess proliferation. Simultaneously, there may be a risk that these immature cells can progress to neoplastic cells if further genetic injury occurs. In colorectal or lung cancer cells, DSC3 was down-regulated by p53-transfection or EGFR-upregulation[12,34]. Taking these reports into consideration, it seems that DSC3 negative phenotype of basal cells can be caused by excess proliferation.
Ki-67 indices of each parameter demonstrated very interesting results as shown in Fig. 7. The value of DSG1 positive cases was significantly higher than that of DSG1 negative cases. Furthermore, the values of cluster 1 and cluster 3 comprising DSG1 positive and negative cases, respectively, were significantly different. It seems that DSG1 plays an important role in keratinocyte proliferation. Although both clusters had K19 negativity in common, the Ki-67 index value of cluster 3 was significantly lower than that of cluster 2, the normal phenotype. In the cells with DSG1−/K19− phenotype, the keratinocytic proliferation seemed to be rather suppressed even though morphological features were hyperplastic. On the other hand, the value of cluster 1 was not significantly higher than that of cluster 2. These results might represent that the proliferation activity in regeneration was limited within a physiological range to avoid excess proliferation.

The cell proliferation, differentiation and cell survival and death is regulated through several pathways. ERK1 and ERK2 are related protein-serine/threonine kinases that participate in the Ras-Raf-MEK-ERK signal transduction cascade, which regulates the keratinocytic proliferation 25). After cell renewal, proliferated cells go into terminal differentiation pathway of keratinocytes towards superficial cells or parakeratotic layers. As described above, DSG1 negative basal cells express EphA2 that facilitates entry into a terminal differentiation pathway, when the EGFR-Erk1/2 signaling is upregulated 26,27). To regulate cell proliferation, EGFR signaling pathway has to be dampened by some molecules. DSG1 and Erbin binding to DSG1 suppress EGFR-extracellular signal-regulated kinase signaling by inhibition of Ras-Raf coupling or EGFR neddylation 28,29). Considering these molecular backgrounds, it seems that the Ki-67 and DSG1 positive basal cells get out from the cell proliferation pathway and enter into the differentiation stage after cell division.

Because our investigation only provides the limited data focusing on cellular phenotypes, some EGFR-related molecules should be examined using current specimens. However, our data are able to confirm the basic phenomena of oral epithelial regeneration through not in vitro or cell culture systems but in vivo. We believe that our results provide the basic information relevant to immunohistochemical examinations in surgical pathology of oral mucosal lesions.

In conclusion, our study showed that regenerative changes such as cell proliferation or epithelium thickening accompanied phenotypic alterations of the epithelium covering mucous cysts. Especially, basal cells alter their characters from the basal cell nature of “DSG1−/ K19+” to the parabasal cell nature of “DSG1+/K19−” during cell growth. Our results show that the alteration from DSG1 negative to DSG1 positive could be crucial for cell proliferation, accompanied by K19-loss, which is consistent with the effects of regulatory molecules such as SIRT2 or EphA2 following EGFR-upregulation. We have to aware that the loss of basal cell character with DSG1 and K19 alterations is basic phenomenon in pathological regeneration, and abnormal DSC3-loss also infrequently occurs in non-neoplastic conditions.

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Conflict of Interest
The authors have declared that no COI exists.

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