A Histopathological and Immunohistochemical Study of Basal Membrane Alterations, with Special Reference to Advance of Dysplastic Grading

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Abstract

A histopathological and immunohistochemical study was conducted to elucidate the alteration of the basement membranes (BMs) and important related factors in oral precursor lesions and oral squamous cell carcinoma (OSCC). Gingivae from 36 cases (5 normal epithelium, 7 hyperkeratosis, 7 mild dysplasia, 7 moderate dysplasia, 5 severe dysplasia, 5 OSCC) were examined. As for histopathological findings of BMs, periodic acid-methenamine-silver stain (PAM) displayed continuous liner appearances in all cases except for OSCC. Reticulin silver impregnation showed discontinuous and irregular distributions were admitted in severe dysplasia and OSCC. From immunohistochemical analysis, laminin 5 γ 2 and collagen IV appeared thicker and more irregularity attitude in moderate, severe and OSCC, while thinner, linear and continuous appearance in control, hyperkeratosis and mild dysplasia. As for integrin α 6, it was increased obviously from hyperkeratosis to mild dysplasia, but it was decreased in severe dysplasia and OSCC on the contrary. Expression of tenascin was gradually increased underlying BMs from hyperkeratosis to OSCC. As for oral precursor lesion, instability of BMs components and up-regulation of BMs related proteins have reinforced from moderate dysplasia. Based on these results, it was supposed that moderate dysplasia became the switch to malignant transformation occurs.

Keywords :

precursor lesion, oral dysplasia, leukoplakia

Introduction

Leukoplakia has been reported wide range of malignant transformation rates as 3-28% (1). The proportion of epithelial dysplasia was about 90% in leukoplakia with malignant transformation, and the transformation rates were raised accompanying with advance of dysplastic grading (2). Oral epithelial precursor lesions are defined as altered epithelium with an increased likelihood for progression to oral squamous cell carcinoma (OSCC) (3). The epithelium of precursor lesion is generally thickened with hyperkeratosis, which is so-called "leukoplakia" and divided into 4 categories as without, mild, moderate and severe dysplasias. Consequently, the name of "leukoplakia" that had been widely used was disappeared, and histopathological dysplastic grading came to be as a disease name (4).

The basement membranes (BMs) is the highly

specialized structure of extracellular matrix between epithelium and underlying connective tissue that consists of a dense network of laminin, collagen IV, integrin and perlecan and some other molecules (5). In neoplasia, alteration and defects of BMs were thought to be associated with the invasive behavior of tumors, including OSCC (6, 7). BMs components were not only degraded during tumor progression, but also newly synthesized and deposited at the tumor invasive front (8). Alterations in the distribution and defects of laminin and collagen IV were particularly indicated parallels the progression of the neoplastic transformation process in oral epithelium (6, 7, 9, 10). However, most of these reports were fragmentary, because only two or three proteins associated with BMs were studied. Few reports concerning about relationship among BMs, cell adhesion to laminin which mediated by integrins (11) and

tissue restructures events which expressed tenascin (12) were reported. The comprehensive study of BMs in dysplastic grading has not been conclusively performed, as yet. In addition, the report that limits the subject site for the research to gingivae is hardly seen.

The objective of this study was to clarify the interrelationship between oral precursor lesion and BMs alteration with special reference to advance of dysplastic grading.

Materials and Methods

Histopathological specimens

Thirty-six specimens from the gingivae were selected from the pathology files of the Department of Oral Pathology, Nihon University School of Dentistry at Matsudo. They consisted of 5 cases of normal epithelium, 7 of hyperkeratosis, 19 of precursor lesions (7 mild dysplasia, 7 moderate dysplasia, 5 severe dysplasia), and 5 of OSCC, with the entire site being in the gingivae. One set of the sections was stained with hematoxylin and eosin (H.E.) and reviewed by oral pathologists to confirm the diagnosis and observe the histological characteristics, and the another set was used for PAM and reticulin silver impregnation and immunohistochemistry. The degrees of epithelial dysplasia were carried out according to the World Health Organization guidelines (3). In addition, the cases secondarily accompanied inflammations were excluded. The protocol was approved by the Committee on Studies Involving Human Beings of Nihon University School of Dentistry at Matsudo (E5.002). Informed consent was obtained from all patients before retrieving the pathological specimens.

Immunohistochemical assessment

Immunohistochemical studies were conducted using formalin-fixed, paraffin-embedded tissue from all cases. Sections (4 μ m thick) were deparaffinized in xylene, and hydrated in graded ethanol solution. The EnVision+ Polymer System (Dako Glostrup, Denmark) was used for antigen detection. Primary antibodies used were directed against the following

antigens: laminin-5 gamma-2 chain (laminin 5 γ 2, 4G1, 1: 40; Dako Glostrup, Denmark); collagen IV (CIV22, 1: 70; Dako Glostrup, Denmark); integrin α 6 (MP 4F10, 1: 100; abcam, England); tenascin-c (tenascin, DB7, 1: 100; BIOHIT, Finland). Antigen retrieval for integrin α 6 was done using citrate buffer solution (pH 6.0), laminin 5 γ 2 and tenascin were done using citrate buffer solution (pH 9.0, Dako Glostrup, Denmark) by pressure pot. For collagen IV was done using proteinase K (Dako Glostrup, Denmark). 0.3% hydrogen peroxide in methanol was used for block endogenous peroxidase activities. The sections were developed in a solution of 3,3'-dianibobenzidine tetrahydrochloride (DAB). Finally, all sections were counterstained with Mayer's hematoxylin. Positive controls for collagen IV and laminin 5 γ 2 were specimens of OSCC of endocervix, and for integrin α 6 and tenascin were specimens of inflammatory granulation tissue. For evaluation of the immunohistochemical staining technique, as a negative control, mouse and rabbit universal negative controls (Dako Glostrup, Denmark) were used during the staining procedure instead of primary antibodies.

Results

Histopathological findings

In Table 1 is summarized the histopathological and immunohistochemical findings. Representative images of oral mucosal lesions, including normal control tissues, are shown in Fig. 1-6. In hyperkeratosis, thicken keratinized layer with acanthosis without cellular atypia (Fig. 2a); in mild dysplasia, general architectural disturbance limited to the lower third of the epithelium accompanied by minimum cellular atypia (Fig. 3a); in moderate dysplasia, general architectural disturbance extending into the middle third of the epithelium (Fig. 4a); in severe dysplasia, greater than two third of the epithelium showing architectural disturbance with associated with cellular atypia (Fig. 5a) were shown. In OSCC with early invasive type, cancer cells with squamous cell characteristics were proliferating and invading into superficial subepithelial zone (Fig. 6a).

Figs. 1-6. Histopathological and immunohistochemical findings in normal mucosa (1), precursor lesions in 4 dysplastic grading : hyperkeratosis (2), mild dysplasia (3), moderate dysplasia (4), severe dysplasia (5), and OSCC (6). a : Histopathological appearances indicated an indistinct/discontinuous part of BM in severe dysplasia and completely failure part in OSCC (H.E. stain, $\times 10$). b : PAM revealed BMs with liner black lining in all lesions except for OSCC (PAM, $\times 20$). c : Reticular fibers stained black was clear in normal, hyperkeratosis and mild dysplasia, though discontinuous and irregular running were admitted in severe dysplasia and OSCC (Reticulin silver impregnation, $\times 20$). d, e : Laminin 5 γ 2 (d) and collagen IV (e) immunoreactivity showed stronger and irregular in the BMs in moderate, severe and OSCC ($\times 20$). f : Integrin α 6 positivity showed the most intensive in mild dysplasia, and decreased in moderate, severe dysplasia and OSCC ($\times 20$). g : Tenascin positivity was appeared gradually stronger with dysplastic grading under BMs ($\times 10$).

Table 1. Result of histopathological and immunohistochemical stainings

Case	No.	PAM	Silver*	Laminin5 γ 2		Collagen IV		Integrin α 6	Tenascin
				Basement membrane		Basement membrane		Basal layer	Under the BM'
Normal	5	weak liner	clear liner	$\pm$	continuous	$\pm$	continuous	$\pm \sim +$	$- \sim \pm$
Hyperkeratosis	7	liner	clear liner	$\pm \sim +$	continuous	$\pm \sim +$	continuous	+	$\pm \sim -$
Mild dysplasia	7	liner	clear thick liner	$\pm \sim +$	continuous	$\pm \sim +$	continuous	++	$+ \sim \pm$
Moderate dysplasia	7	irregular liner	liner/partial indistinct	$\pm \sim +$	discontinuous	+	continuous	$+ \sim ++$	$+ + \sim +$
Severe dysplasia	5	weak liner	discontinuous	$+ + \sim +$	discontinuous	$+ \sim ++$	continuous	$+ \sim \pm$	++
OSCC	5	weak discontinuous	discontinuous	++	discontinuous	$+ \sim ++$	discontinuous	$+ \sim \pm$	++

++ : strong positive, + : moderate positive, $\pm$: weak positive, - : negative

Silver* : reticulin silver impregnation

BM' : basement membrane

Concerning about BMs, indistinct and completely failure parts were admitted in severe dysplasia and OSCC with H.E. staining, respectively (Figs. 5a and 6a). In the special staining procedure, PAM displayed continuous liner appearances in all cases except for OSCC (Fig. 6b). As for reticulin silver impregnation, running of reticular fibers stained black as supporting BMs was clear in normal, hyperkeratosis and mild dysplasia (Figs. 1c-3c), though partial indistinct parts were observed in moderate dysplasia (Fig. 4c). In severe dysplasia and OSCC (Figs. 5c and 5d), discontinuous and irregular running of reticular fibers were admitted under the BMs, on the contrary.

Immunohistochemical staining

Laminin 5 γ 2 stained stronger, thicker and more discontinuous attitude in the BMs in moderate, severe dysplasias and OSCC (Figs. 4d-6d), while thinner, linear and continuous appearance in control, hyperkeratosis and mild dysplasia (Figs. 1d-3d). Concerning about collagen IV, it showed stronger attitude in moderate, severe dysplasias and OSCC (Figs. 4e-6e) and more discontinuous appearance was in OSCC (Fig. 6e), linear and continuous appearance in control, hyperkeratosis and mild dysplasia (Figs. 1e-

3e), on the contrary. As for integrin α 6, positive findings were increased obviously in the basal and supra-basal layers from hyperkeratosis and mild dysplasia (Figs. 2f and 3f). In moderate, severe dysplasias and OSCC, the distributions of integrin α 6 were decreased and scattered in the basal and supra-basal layers (Figs. 4f-6f). Tenascin in the underlying BM gradually increased from hyperkeratosis to OSCC ; with advanced dysplastic grading (Figs. 2g-6g). More intensive reaction was observed in moderate, severe dysplasias and OSCC (Figs. 4g-6g), in particular.

Discussion

Interactions between tumor cells and the remodeling of extracellular matrices (ECMs) ; failure of BMs (13) and alteration of ECMs molecules (14, 15, 16) are crucial determinants of tumor cell proliferating and transforming activity. But in the oral lesion, the research on the alteration of BMs environment with the advance of dysplastic grading was not enough, though lots of previous reports mainly retrieved about collagen IV and laminin (13, 17). In addition, the report that limits the subject site for the research to gingivae is hardly seen. Oral mucosae are

divided into masticatory and non-masticatory mucous membranes, and the characteristics of the mucous epithelium and the connective tissue have different according to each region.

As for histopathological findings of BMs, PAM displayed continuous liner appearances in all cases except for OSCC. Reticulin silver impregnation showed discontinuous and irregular distributions were admitted in severe dysplasia and OSCC. That is, the disorder of running of reticular fibers as supporting BMs was clear in these lesions. These histopathological findings were corresponding to diagnostic criteria and biological attitudes (3). Combination of H.E., PAM and reticulin silver impregnation was very useful for the judgment of micro invasion.

Laminin is an extra-cellular protein with heterotrimer molecule with an $\alpha 3$, $\alpha 5$, $\beta 1$, $\beta 3$, $\gamma 1$ and $\gamma 2$. Normally, constituents of laminin-5 were expressed in squamous epithelium (18), and laminin 5 $\gamma 2$ was considered the most important anchoring filaments fixing basal keratinocytes to the BMs via integrins to hemidesmosomes as mechanical resistance and protective function in particular (18, 19). Tosios KI *et al.* described that alterations of collagen IV and laminin 5 $\gamma 2$ distributions in precursor lesions showed parallel with the advance of dysplastic grading (6, 7). Bryne M *et al.* noted that no correlation was found between expressions of these proteins and grade of malignancy (20), on the contrary. In this study, moderate, severe dysplasia and SCC showed similar immunoreactivity with collagen IV and laminin 5 $\gamma 2$ as emphasized appearances. However, the advance of dysplastic grading was not completely parallel with these immunopositive distributions. The over-expression of laminin 5 $\gamma 2$ and collagen IV might be as a result of fragmentation generated by BMs degradation during tumor proliferating. And these results were suggested that new deposition of major BMs proteins could function as an extracellular ligand helping migration and invasion of tumor cells.

Integrins are a family of transmembrane receptors that mediate both cell-cell and cell-matrix adhesion. They are heterodimers composed of an α and a β

transmembrane glycoprotein subunit with 22 different integrins are found in nature (21). Integrin $\alpha 6$ complexes are receptors for laminin, which play important roles in regulating tumor malignant transformation (21). Stratified squamous epithelium expressed a range of integrins including $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 4$ (22). Only $\alpha 6\beta 4$ was concentrated in basal cells adjacent to the BMs (22), and was expressed in the gingiva with polarization towards the BMs as a liner band (23). Furthermore, integrin $\alpha 6\beta 4$ was shown during tumor progression and wound healing in oral precursor lesions (24), was correlated to loss of BMs related proteins (25). There was emphasis of basal integrin expression in hyperkeratosis and mild dysplasia where the BMs remained intact. It was suggested that this emphasis might be resistance from proliferating basal epithelial cells (2).

The fragmentation and new deposition of BMs related proteins were coincident with loss of integrin $\alpha 6$ in moderate, severe dysplasias and OSCC in the present study. Lack of restricted basal polarization of integrin $\alpha 6$ might be an early marker of oral malignant transformation including abnormal signals from the ECMs. Consequently, it was suggested that multiple disorganization of the BMs in moderate, severe dysplasias and OSCC caused by proliferation of epithelial cells (2) and activation of integrin. Previous reports described that the expression of unstable BMs related proteins as biological markers for assessing oral carcinogenesis (13, 17, 22), and it was degradation and/or loss is the first stage of invasion.

The inflammatory reactions of the subepithelial zone were increased with the advance of dysplastic grading, and were marketable in severe dysplasia and SCC (26). In the extrinsic pathway, cancer related inflammation is accompanied by tissue remodeling including failure of BMs. On the other hand, inflammatory cells can cause break the BMs (27), and inflammatory cytokines can potently upregulate tenascin expression secreted by mesenchymal cells (28), keratinocytes, and SCC cell lines (29) as inflammatory reaction. Tenascin is a large glycoprotein component of ECMs with a molecular

weight of 190–250 kD (30), and is gradually appeared underlying BMs associated with the advance of dysplastic grading in the present study. It is possible that basal keratinocytes uncontrolled increase in proliferation and failure of BMs may secrete soluble factors that in turn may stimulate inflammatory cells and stromal fibroblasts to organize high levels of tenascin (31).

The mechanisms of BMs instability/loss were suggested that due to insufficiency, synthesis and increased turn over or degradation of BMs components by the tumor cells (32). As for oral precursor lesions, instability of BMs components and up-regulation of BMs related proteins has reinforced from moderate dysplasia. Consequently, it was supposed that moderate dysplasia became the switch to malignant transformation occurs from the above-mentioned result.

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