Oral Candidiasis: A Histopathological, Ultrastructural and Immunohistochemical Study

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Abstract

Oral candidiasis presents characteristic histopathological findings. The mucous epithelium at the site of candidiasis is covered by a hyperkeratinized layer. But, the factors leading to hyperkeratosis in candidiasis were unknown. To investigate the factors which led to hyperkeratosis in oral candidiasis, we histopathologicaly, histochemicaly, and ultrastructualy observed the morphological alteration of the epithelial tissue, and studied the factors in connection with those changes immunohistochemically.

Candidiasis group (8 cases of papilloma, 5 cases of verrucous hyperplasia), non-candidiasis group (8 cases of papilloma, 7 cases of verrucous hyperplasia), and 5 subjects with normal oral mucosa were selected. Ultrastructually, *Candidal* hyphe internalized into the epithelial cytoplasmic vacuole resembling endocytosis. Destruction of desmosomal cell junction and deficit of tonofilament was also observed at the tip of the hypha. Immunohistochemistry, weaker expression for E-cadherin was seen in the candidiasis group. Strong positive E-cadherin reaction was seen along the insertion sites of the hyphae. CK13 was negative in non-candidiasis group, and weakly positive in candidiasis groups in the basal layer, respectively. Weakly positive EGFR reactions were observed in the prickle layer in candidiasis group. Inflammatory reaction

with NE, CD68, COX-2, CD105 and Ki-67 were markedly observed in candidiasis group.

The above result suggested that the hyperkeratosis in oral candidiasis was related with E-cadherin in connection with adhesion and penetration of *Candidal* hyphae and the precocious expression of the stratified squamous epithelium later differentiated marker CK13. Furthermore, it was surmised that inflammatory response by *Candidal* hyphae invasion itself led to hyperkeratosis.

Introduction

Oral candidiasis is the most frequently encountered fungal infection caused by *Candida* species (spp.), and may be either endogenous mycosis or an opportunistic infection (1–4). Oral candidiasis presents a diverse range of clinical features; pseudomembranous, erythematous, erosive or hypertrophic (5). It is not uncommon for oral candidiasis to be discovered by chance in tissue resected surgically from patients with oral mucosal disease. With respect to these factors, the pathology of candidiasis is complicated by the diversity of host immune deficiencies and the different types of cytotoxicity of *Candida* spp. (6).

Candidiasis presents characteristic histopathological findings. *Candida* spp. characteristically show invasion vertically from the keratinized layer to the prickle layer, and invasion has three stages; the yeast adheres to the epithelium, followed by budding yeast, and then invasion of the epithelium (7). The mucous epithelium at the site of the candidiasis is covered by a hyperkeratinized layer, and the *Candida* hyphae is not reaching deep into the prickle layer (8-12). The ability of *Candia* spp. to proliferate in the mucosal tissue may be dependent on hyperkeratosis (13). There have been some reports ultrastructual or histopathological studies of morphological changes in the oral mucous epithelium

accompanying invasion by hyphae (14-16). However, the consideration in connection with the mechanism which led to hyperkeratosis was not described. The factors leading to hyperkeratosis in candidiasis were unknown (13).

If *Candida* penetrates the surface of the mucous epithelium and reaches the prickle layer, inflammatory cell infiltration may be found and there may be formation of intraepithelial microabscesses. At this stage, cytokines are produced as a result of interaction between *Candida* and its receptors present on epithelial cells, and the physical stimulus caused by *Candida* adhering to and penetrating the epithelium provokes inflammation of the mucous membrane (17-19). Nevertheless, objective evaluation of inflammatory response in oral candidiasis was not performed, and the relevance of hyperkeratosis was ambiguous.

To investigate the factors which led to hyperkeratosis in oral candidiasis, we histopathologicaly, histochemicaly, and ultrastructualy observed the morphological alteration of the epithelial tissue, and studied the factors in connection with those changes immunohistochemically.

Materials

Subjects were 2,905 cases of oral mucous disease that underwent surgical resection and histopathological diagnosis at the Hospital of Nihon University School of Dentistry at Matsudo from 1976 to 2012. They were consisted of 677 cases of fibro-epithelial polyps, 1,388 cases of leukoplakia, 569 cases of papilloma, 109 cases of pyogenic granuloma, 28 cases of verruciform xanthoma, 87 cases of verrucous hyperplasia, and 47 cases of verrucous carcinoma.

Methods

1) Histopathological and histochemical studies

Each case was observed for the presence of fungus exhibiting a reddish violet color on PAS reaction, and those cases in which intraepithelial fungal invasion was found were regarded as the *Candida* infection group. All 2,905 cases were divided into the with candidiasis and without candidiasis groups. In candidiasis group, the form of the invasion of *Candida* hyphae into the mucous epithelium and the inflammatory response of the subepithelial connective tissue were reconfirmed microscopically using HE-stained specimens.

For mitosis index (MI), an arbitrary field was photographed under optical microscopy at

400× magnification, and the number of cells in the late or final mitotic phase was counted. MI was calculated as the percentage of mitotic cells from a total of 1,000 or more cells.

2) Transmission electron microscopical study (TEM)

Each 2 cases of papilloma with or without candidiasis were used for the ultrastructual study. Parts of the specimen fixed with 10% neutral formalin were washed and refixed with 2% glutaraldehyde and 1% osmium tetroxide. The tissue was then embedded in epoxy resin (Quetol 812, Nisshin EM, Tokyo, Japan) by the usual method. Some of the blocks selected were cut using ultramicrotome at 60-90 nm with a diamond knife. Ultrathin sections were poststained with uranyl acetate and lead citrate, and observed under a TEM (JEM-1200 EX Ⅱ, JEOL, Tokyo, Japan).

3) Immunohistochemical study

The selection criteria for the subjects were as follows. Exclusion criteria were precancerous lesion, malignant tumor and ulcer or erosion formation and inclusion criteria were the cases in which the same pathological diagnosis including more than 5 cases both with or without

candidiasis. On this basis, 8 cases of papilloma and 5 cases of verrucous hyperplasia were selected from the candidiasis group. Controls were 8 cases of papilloma, 7 cases of verrucous hyperplasia without candidiasis groups, and 5 subjects with normal oral mucosa. Subjects for immunohistochemical study were shown in Table 1.

Immunohistochemical studies were conducted using 10% neutral formalin solution-fixed, paraffin-embedded tissues. Sections were deparaffinized in xylene and hydrated in graded ethanol solution. EnVisuon + Polymer System (Dako Glostrup, Denmark) was used for antigen detection. Primary antibodies and antigen retrieval methods used in this study were shown in Table 2. Secondary antibody reaction was carried out using EnVision + Polymer System Dako Glostrup, Denmark). Antigenic reactions were detected using 3,3'-dianibobenzidine tetrahydrochloride (DAB) and then counterstained with Mayer's hematoxylin.

In addition, as positive controls for each antigen, oral squamous carcinoma was used for Ki-67, EGFR, COX-2, NE and CD105. Epithelial hyperplasia was used for CK13 and E-cadherin, and verruciform xanthoma was used for CD68. For the negative control, IgG1 negative control (dilution: 1: 100, DakoCytomation, Glostrup, Denmark) was used in place of the primary antibodies Ki-67, CD68, CD105, E-cadherin, EGFR, COX-2 and NE, while IgG2a

negative control (dilution: 1:50, DakoCytomation, Glostrup, Denmark) was used in place of the primary antibody CK13.

The assessment of the immunohistochemical result was performed combining the quantitative concentration analysis which used Win ROOF Version 3.4 (Mitani Corporation) image analysis software and microscopic observation. As for the judgment of the protein revelation level for E-cadherin, CK13, EGFR, and COX-2 was as follows, ++: strong positive (130 or less), +: moderate positive (131-180), ±: weak positive (181-220), and -: negative (221 or more). The judgment of NE and CD68 was done by the number of the positive cells stained blackish brown; ++: high density, +: large number, ±: small number, and -: nothing.

4) Quantitative morphological study

For the proliferative index (PI), an arbitrary field was photographed under optical microscopy at 400× magnification, and cells with nuclei stained dark reddish-brown for ki-67 were considered positive. PI was calculated as the percentage of positive cells from a total of 1,000 or more cells.

For capillary vessels, the cell membrane of vascular endothelial cells was stained dark

reddish-brown using CD105 antibody, and those forming a luminal structure were examined under optical microscope at 400× magnification, and the distance between the basal membrane and the blood vessel (μm) and the blood vessel area (μm^2) were measured using Win ROOF Version 3.4 (Mitani Corporation) image analysis software. Mean basal membrane−blood vessel distance and mean blood vessel area was calculated for each case from an arbitrary field photographed under optical microscope.

5) Statistical study

Tests for statistically significant differences were carried out using statistical testing software (SPSS 11.0J). Morphometrical differences were tested with Mann-Whitney U-test, and MI and PI were tested by Chi-square test. Statistical significance was set at $p<0.05$.

Results

1) Histopathological and histochemical studies

Histopathological findings are shown in Fig.1 and Table 3. With parakeratotic stratified squamous epithelium, there was outward papillary proliferation in papilloma (Fig.1-a) and

outward verrucous proliferation in verrucous hyperplasia (Fig.1-c). In either case, narrow connective tissue was the axis for epidermal growth. In the candidiasis group, yeast cells adhered to the surface of the epithelium, and pseudohyphae or hyphae penetrated vertically into the keratinized layer or upper layer of the prickle layer (Figs.1-b,d). In the outer surface of the mucous membrane and connective tissue, slightly to moderately neutrophilic infiltration was observed, and inflammatory cell invasion of the epithelium were observed (Fig.1-d). PAS reaction revealed clearly hyphal invasion (inset in Fig.1-b and Fig.1-d).

Comparing MI by the presence or absence of candidiasis, MI was greater in both papilloma and verrucous hyperplasia cases in the candidiasis group. Particularly in papilloma cases, a significant difference was found (papilloma without candidiasis, MI=0.6; papilloma with candidiasis, MI=1.4; $p<0.001$).

2) Transmission electoron microscopical study (TEM)

Ultrastructual findings are shown in Fig.2. Ultrastructually, longitudinal section of *Candidal* hyphae (HL) was existed within the oral epithelium (E). Cross section of *Candidal* hyphae (HC) internalized into the epithelial cell inside a cytoplasmic vacuole (V), resembling

endocytosis. The cell covered with plasma membrane (PM) is surrounded by thick cell wall (CW). (Fig.2-1:x5,000). *Candial* hyphae containing mitochondrias (M) and a nuclear (N) had invaded along an intercellular bridge was observed. Desmosomal cell junction (D) of the epithelial cells (E) and dense tonofilament (TF) were arranged irregularly in the host cytoplasm. Destruction of desmosomal cell junction (arrow) and deficit of tonofilament (arrowhead) were also observed at the tip of the hypha (Fig.2-2:x10,000).

3) Immunohistochemical study

Immunohistochemical results are shown in Tables 3 and 4, and representive findings are presented in Figs.3 to 5.

E-cadherin was positive in the basal layer of normal mucous membrane, papilloma and verrucous hyperplasia, but the level of expression was weaker in the candidiasis group than in the without candidiasis group. In the without candidiasis group, a strong positive reaction was seen across the whole of the prickle layer, which presented a clearly scalar appearance (Figs.3-a,c). In the candidiasis group, weaker expression was seen in the prickle layer, and the reduction was particularly marked in the upper layer (Figs.3-b(1),d(1)). On the surface of the

keratinized layer where *Candida* fungal invasion was obvious, strong positive E-cadherin reaction was seen along the insertion sites of the hyphae (Figs.3-b(2),d(2), arrowhead).

CK13 was negative in the basal layer of normal mucous membrane, papilloma and verrucous hyperplasia without candidiasis (Figs.4-a1,c1). In normal mucous membrane, CK13 was weakly to moderately positive in the lower of the prickle layer and moderate positive in the upper layer. In papilloma and verrucous hyperplasia with candidiasis, CK13 was weakly positive in the basal layer and strongly positive in the whole prickle layer. In the prickle layer, the positive reaction was weaker in the candidiasis group than the without candidiasis group, with weak and strong positive reactions found mixed together (Figs.4-b1,d1). About candidiasis group, comparative observation of the staining attitude of the *Candidal* penetration site and the non-penetration site close to it were performed. In *Candidal* penetration site, decreased CK13 and E-cadherin reaction in the prickle layer. Concerning about non-penetration site, positive for CK13 and E-cadherin in the prickle layer was observed.

In the investigation of cell proliferation using Ki-67 antibody, higher values were found in both the basal layer and the prickle layer of the candidiasis group (Figs.4-b3,d3) than the without candidiasis group (Figs.4-a3,c3). With verrucous hyperplasia, both the basal layer and

the prickle layer showed significantly higher values in the candidiasis group (Fig.4-d3, basal layer, 32.7%; prickle layer, 0.7%) than the without candidiasis group (Fig.4-c3, 12.1%, 0.1%) ($p<0.001$).

EGFR was almost completely negative in the basal layer and lower of the prickle layer in both the candidiasis and without candidiasis groups (Figs.4-a2,b2,c2,d2). Weakly positive EGFR reactions were observed in the upper layer of the prickle layer in papilloma and verrucous hyperplasia with candidiasis (Figs.4-b2,d2).

NE was almost completely negative in both the epithelium and connective tissue in normal mucous membrane and the without candidiasis group (Figs.5-a1,c1). In papilloma and verrucous hyperplasia with candidiasis, strongly positive reactions were seen on the surface of the epithelium, and positive reactions were seen on the prickle layer and connective tissue (Figs.5-b1,d1). CD68 was almost completely negative in normal mucous membrane, papilloma and verrucous hyperplasia without candidiasis (Figs.5-a2,c2). In papilloma and verrucous hyperplasia with candidiasis, however, many strongly positive cells were observed (Figs.5-b2,d2). COX-2 was negative in all layers of normal mucous membrane, papilloma, and verrucous hyperplasia without candidiasis (Figs.5-a3,c3). COX-2 was positive in all layers in

papilloma with candidiasis (Fig.5-b3). In verrucous hyperplasia with candidiasis, weakly positive cells were seen in the basal layer, and the prickle layer showed a mix of strongly and weakly positive cells (Fig.5-d3).

In the investigation of new blood vessels using CD105 (Figs.5-a4,b4,c4,d4), numerous enlarged blood vessels were found in the connective tissue immediately below the basal membrane in papilloma and verrucous hyperplasia with candidiasis (Figs.5-b4,d4). In these lesions, epithelial invasion by capillary vessels was observed. Image analysis showed that the mean membrane−blood vessel distance was significantly shorter in the candidiasis group (papilloma, 86.0 μm; verrucous hyperplasia, 119.7 μm) than in the without candidiasis group (170.8 μm, 409.4 μm) ($p<0.001$). Mean blood vessel surface area at 500 μm below the basal membrane was significantly bigger in the candidiasis group (papilloma, 159.7 μm^2; verrucous hyperplasia, 96.5 μm^2) than in the without candidiasis group (9.3 μm^2, 13.6 μm^2) ($p<0.001$).

Discussion

Candida spp. can be detected in the oral cavity of healthy adults with no clinical symptoms, and the prevalence is reported to be 3-48% (20). Kuyama et al. described that 74.4% of

candidiasis patients are aged 60 years or more (5). In the present study, the candidiasis group tended to have more advanced age than the without candidiasis group.

Oral candidiasis penetrates vertically from the keratinized layer to the upper layer of the prickle layer, with the following stages: adherence of yeast cell to the epithelium; proliferation; establishment; budding yeast; and intraepithelial invasion (6,7,21). Moreover, a histopathological characteristic of oral candidiasis is hyperkeratinization, which is hyperplasia of the surface layer keratinocytes, accompanying invasion by hyphae (10). However, there are few reports on the morphological changes of the mucosal epithelium tissue, which accompany invasion by hyphae (14-16). Moreover, there has yet to be any discussion of the role of *Candida* spp. with respect to differentiation of keratinocytes (22).

We therefore performed ultrastructural and histopathological observations of the relationship between *Candida* spp. and the oral mucosa, and we also performed an immunohistochemical investigation of the morphological changes of the oral mucosal epithelium accompanying invasion by hyphae with special reference to inflammation involving the blood vessels.

Candida spp. may be present as either budding yeast or pseudohyphae. Pseudohyphae invade epithelial cells as if spearing them, and for this reason are known as “shish-kebabs” (23).

In the present study, histopathological and ultrastructural observations revealed that the yeast cells adhered to the surface layer of the epithelium, and pseudohyphae or hyphae penetrated vertically to the keratinized layer or the prickle layer.

The toxicity of *Candida* spp. involves both their adherence and their morphology (24,25). Zhu et al. show that *Candida* spp. penetrates by physically elongating (18), and also that the main adherence molecules are proteins, which induce bonding to E-cadherin on the mucosal epithelial cell surface and thus induce endocytosis (18,26). Candidal hyphale were internalized into the epithelial cell inside a cytoplasmic vacuole, resembling endocytosis in this ultrastructual study.

The morphology of *Candida* hyphae is important for adhering and penetrating epithelial cells (15), and the relationship with E-cadherin in particular has been reported (26). In the present study, a strong positive E-cadherin reaction was found along the hyphae insertion sites in candidiasis group cases where there was clear invasion of the outer surface in the keratinized layer (Figs.3-b(2),d(2)). This finding also suggests that adherence of *Candida* to epithelial cells is mediated by E-cadherin. Moreover, weakly positive EGFR reactions were found in the upper layer of the prickle layer in papilloma and verrucous hyperplasia with candidiasis. EGFR is

reported to induce endocytosis of *Candida* spp. (18), but the mechanism is unclear.

Expression of E-cadherin was weaker in the prickle layer of papilloma and verrucous hyperplasia with candidiasis, and the decrease of reaction was particularly marked in the upper layer (Figs.3-b(1),d(1)). Sitheeque and Samaranayake report that when *Candida* was incubated with keratinocytes in an *in vitro* study, E-cadherin expression gradually decreased (22). The immunohistochemical findings within the same individual in this study were also the result of supporting *in vitro* study (22). *Candida* spp. is able to adhere to cells and to secrete proteases, and it appears that E-cadherin decreased in the region of hyphal penetration because the proteases degenerated the extracellular matrix and adherence molecules (19). Destruction of desmosomal cell junction was also observed ultrastructually at the tip of the hypha in this study (Fig.2-2).

Concerning about epithelial hyperkeratosis, E-cadherin plays a role in inhibiting expression of later differentiated markers in cells of the parabasal layer of stratified squamous epithelium (14,27), so that loss of E-cadherin is believed to be responsible for expression of later differentiated markers (27). Concerning about the basal layers in the present study, the stratified squamous epithelium later differentiated marker CK13 was negative in those of normal mucous

membrane and the without candidiasis group (Figs.4-a1,c1), but showed weak positive reactions in the candidiasis group (Figs.4-b1,d1). Thus, weakened cell adherence may be one of the factors in hyperkeratosis. On the contrary, *Candida* has been shown to stimulate expression of CK13 (22,28), which suggest that *Candida* without E-cadherin is involved in differentiation of epithelial cells. In any case, a relationship between *Candida* and the epithelial cell later differentiation marker CK13 was suggested.

Many researchers have noted that hyperkeratotic layers are the most suitable for *Candida* proliferation in mucosal epithelial tissue, and that *Candida* does not penetrate to the middle of the prickle layer or the basal cell layer (8-12). One reason for this is the presence of the anti-*Candida* protein calprotectin (29) distributed in the lower layer of the prickle layer. Calprotectin is related to middle-stage differentiation of keratinocytes (13). Candidiasis is caused by the sensitivity of keratinocytes and the ability of *Candida* to inhibit calprotectin (22).

In the present study, the investigation of cell proliferation using Ki-67 antibody, the candidiasis group showed higher cell proliferation than the without candidiasis group (Figs.4-b3,d3). Dwivedi reported increased cell cycle markers in an experimental model of candidiasis (30), and Sohnle argued that the reason for this is increased turnover of basal cells

due to precedence of inflammation at the site of infection (31). Sohnle conjectured that chronic inflammation caused secondary thickening of the epithelium, and in the present study as well, there may have been cell-mediated reactions in which cytokines acted on basal cells to increase their differentiation (31).

In the present study, the CK13 reaction was weaker in the prickle layer of the candidiasis group than in the without candidiasis group (Figs.4-b1,d1). These findings indicate that although later differentiated marker expression increased in the basal layer, mature differentiation did not take place in epithelial cells because the cell cycle was accelerated and because of tissue damage caused by hyphal invasion.

Important steps in the adherence of yeast cells to the epithelium are the formation of pseudohyphae and tissue permeability (32). *Candida* spp. adheres to the epithelium and induces endocytosis (18), and pseudohyphae invade from within or between epithelial cells by activating permeability of the cells. Accelerated cell permeability causes considerable damage to the cell.

As for inflammatory response, the majority of *Candida* spp. produce toxicity factors that include protease factors (6), provoking a neutrophil-mediated acute inflammatory response (33).

In the present study of immunohistochemistry, positive NE reactions found in the outer and prickle layers of the epithelial and connective tissue in the candidiasis group. In addition, many cells that showed a strongly positive CD68 reaction were observed in the connective tissue in the candidiasis group (Figs.5-b1,d1,b2,d2). From these findings, it appears that the release of inflammatory mediators from epithelial cells in the infection site induced antifungal responses such as neutrophils and macrophages, which are inflammatory cells that circulate within the tissues (14). On image analysis of new blood vessels using CD105, vessels in the candidiasis group showed significantly greater expansion and hyperplasia, and proximity to the basal lamina, than in the without candidiasis group, indicating a blood vessel reaction caused by candidiasis. Moreover, as images of epithelial invasion of new blood vessels were observed in the candidiasis group, it was conjectured that neutrophils infiltrating from the new blood vessels migrated into the spaces between epithelial cells. It has been stated that the morphological change from yeast cells to hyphae facilitates adherence to epithelial cells and becomes the cue for the inflammatory response (34,35). At the same time, Villar et al. argued that the key to fungal infection is not only the formation of hyphae, but also the host's strong inflammatory response to hyphal invasion (34).

COX-2 is an enzyme induced by growth factor, cytokines, cancer genes, tumor promoters and other such stimuli (36). It is not typically expressed in healthy cells (37), but the expression is seen with inflammatory disease (38). In the present study, a positive COX-2 reaction in the candidiasis group was found in the prickle layer in particular (Figs.5-b3,d3). Deva reported that *Candida* spp. induces COX-2 gene expression and produce prostaglandin E2, and that prostaglandin E2 plays an important role in *Candida* growth (37). *Candida* thus appears to acquire an environment that contributes to its own growth through the expression of COX-2 in cells that it has invaded.

The above result suggested that the hyperkeratosis in oral candidiasis was related with E-cadherin in connection with adhesion and penetration of *Candidal* hyphae and the precocious expression of the stratified squamous epithelium later differentiated marker CK13. Furthermore, it was surmised that inflammatory response by *Candidal* hyphae invasion itself led to hyperkeratosis.

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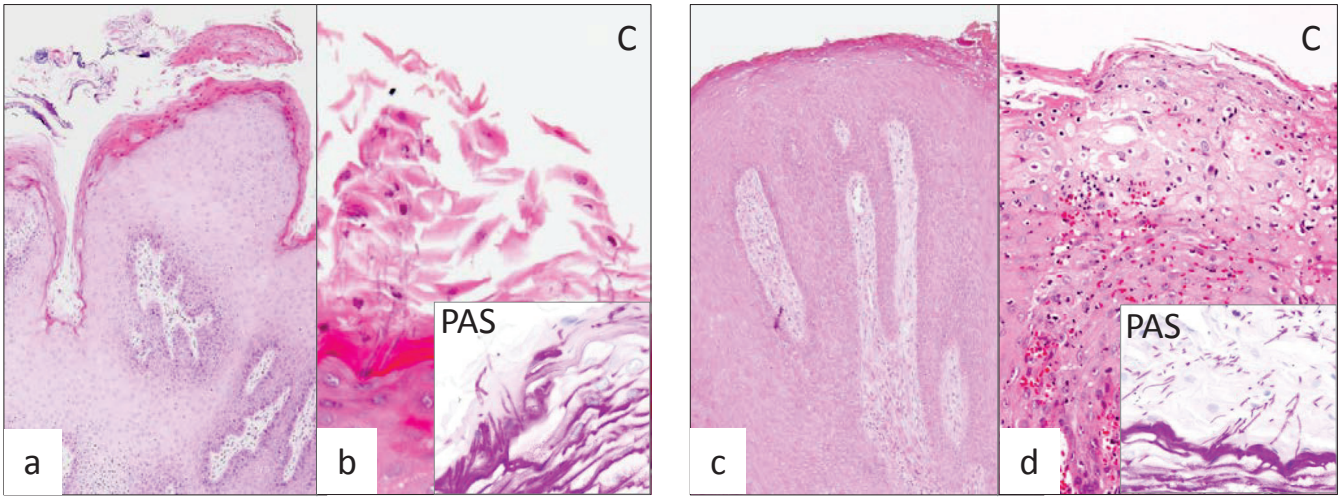

Fig.1

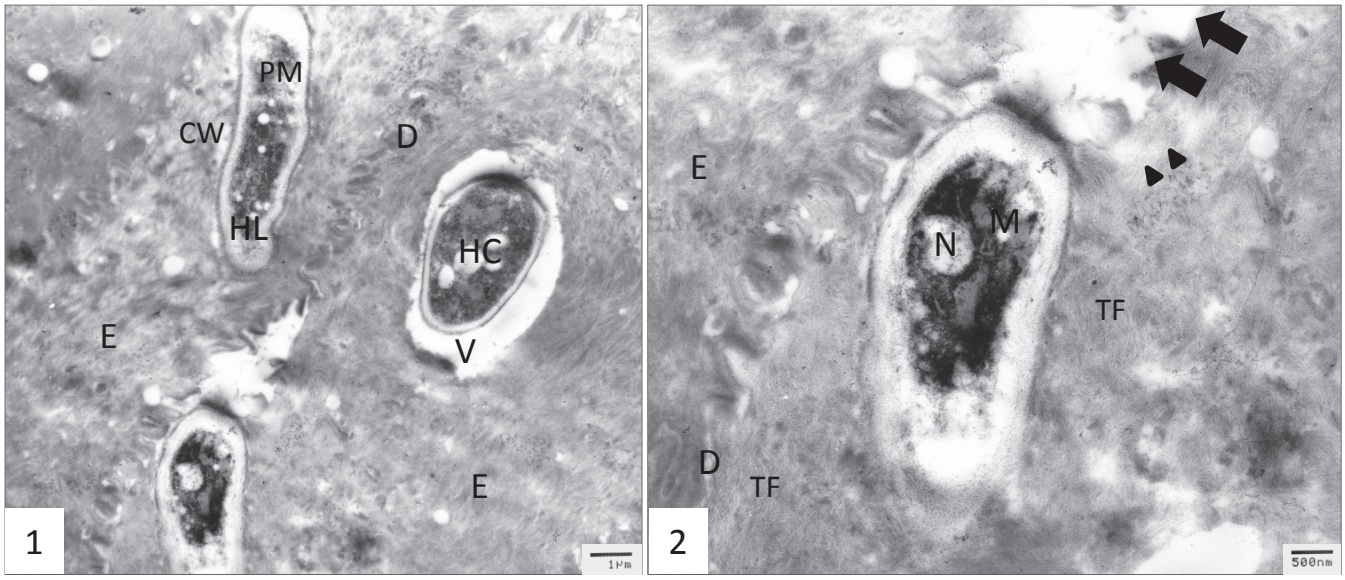

Fig.2

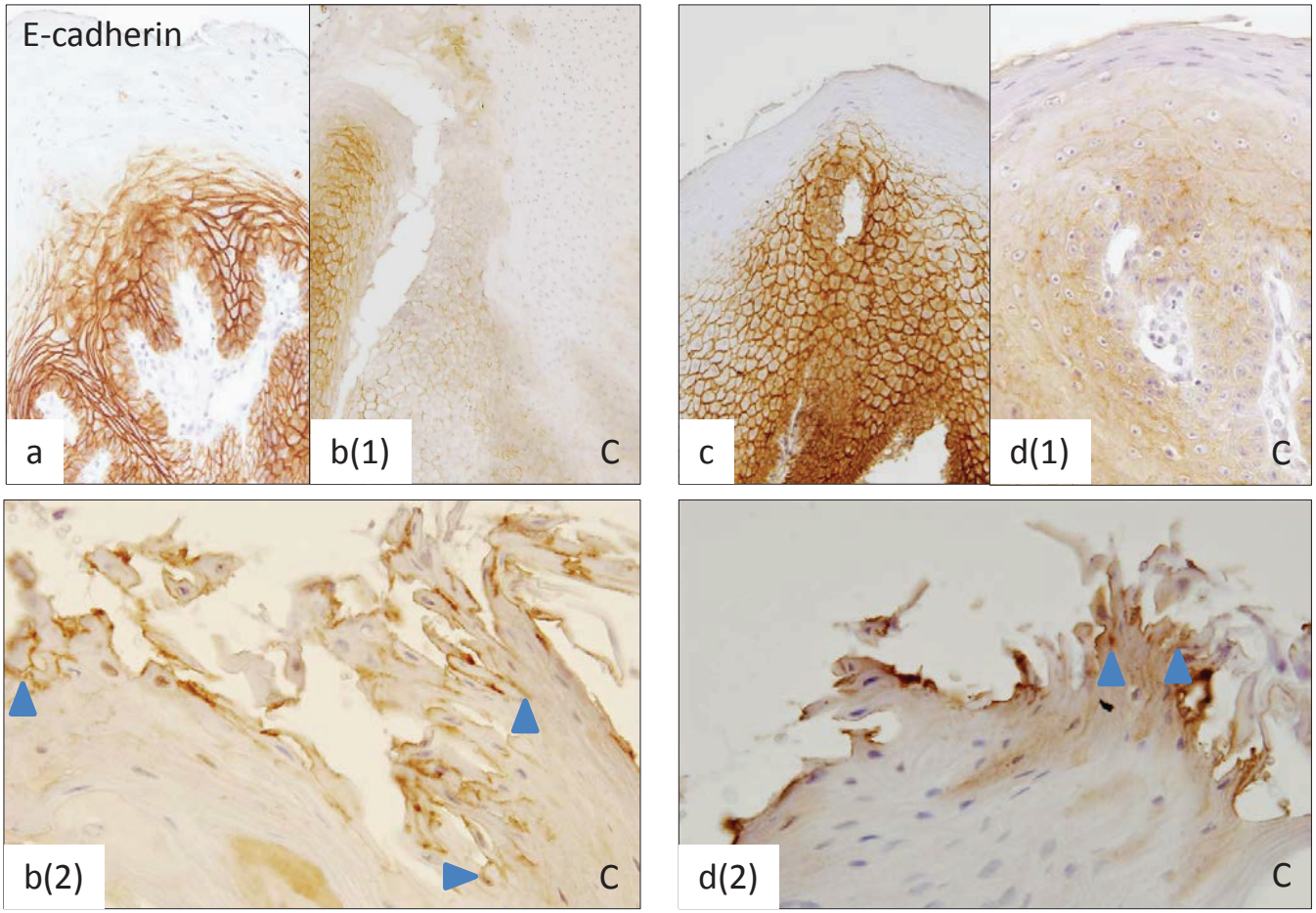

Fig.3

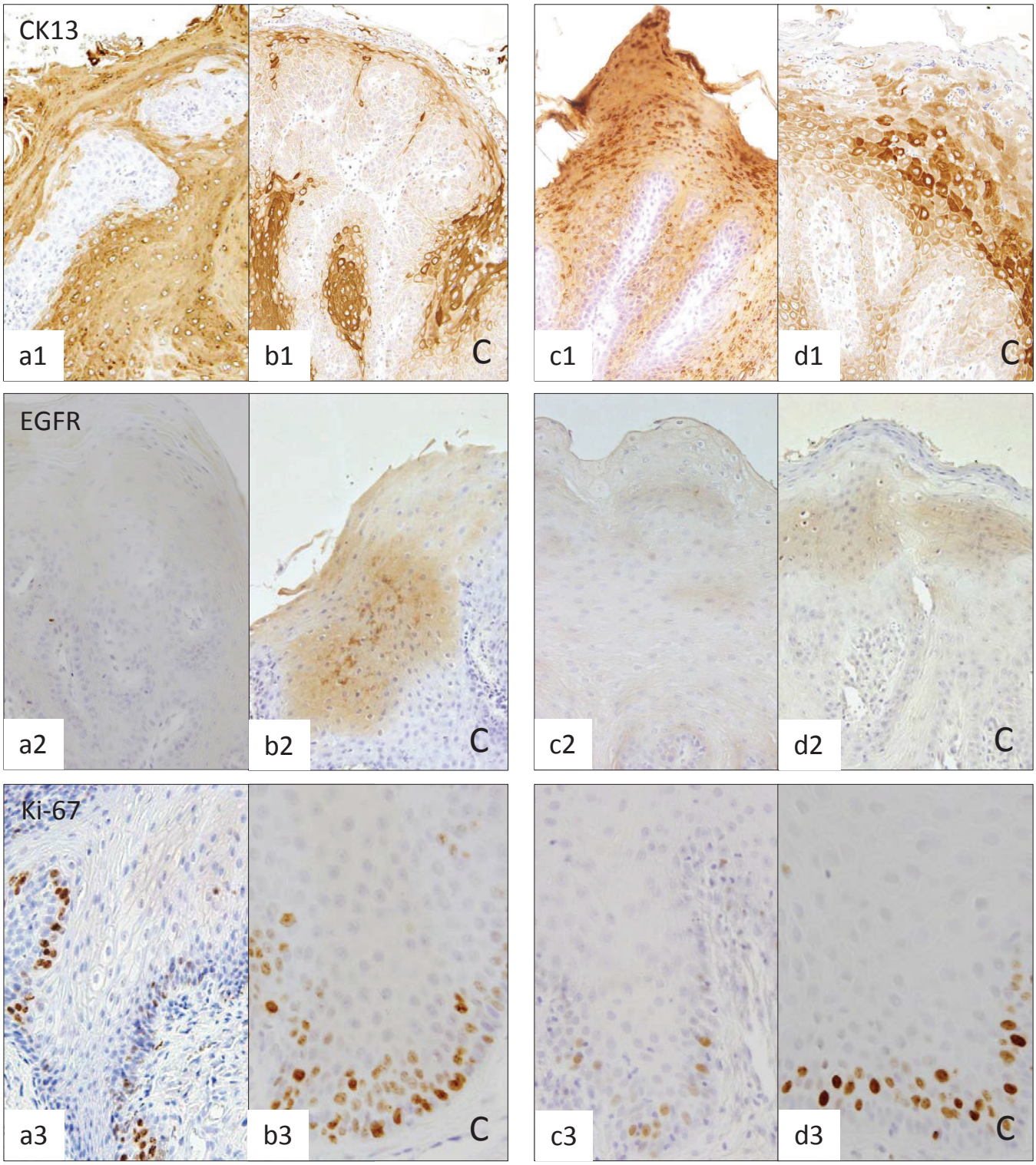

Fig.4

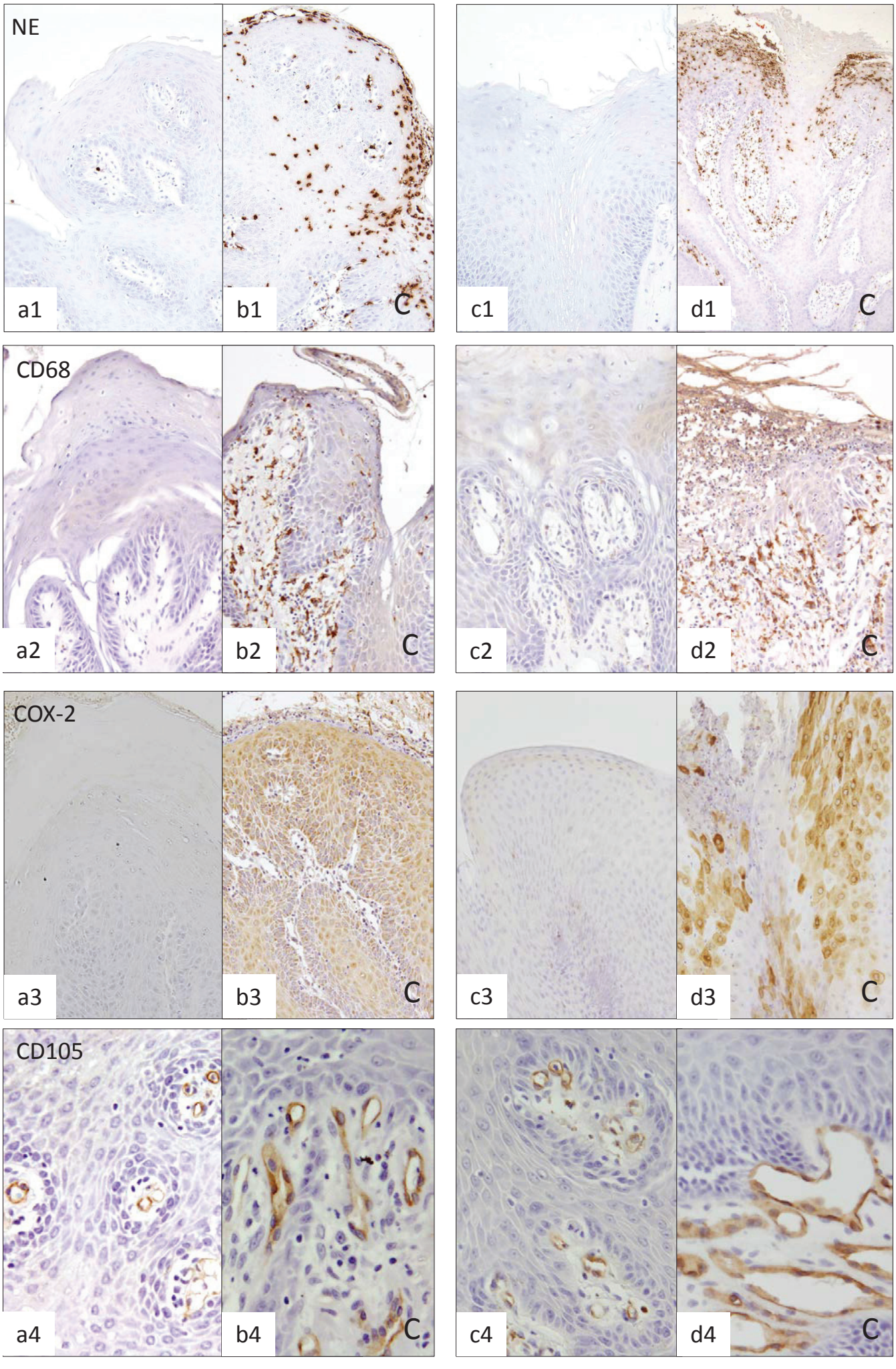
NE
a1
b1
C
c1
d1
C
CD68
a2
b2
C
c2
d2
C
COX-2
a3
b3
C
c3
d3
C
CD105
a4
b4
C
c4
d4
C

Fig.5

Figure 1

Histopathological findings of papilloma (a: without candidiasis, b: with candidiasis) and verrucous hyperplasia (c: without candidiasis, d: with candidiasis). “C” means candidiasis in the figures.

Papillary and verrucous outward proliferation with parakeratotic stratified squamous epithelium was observed in papilloma (Fig.1-a) and verrucous hyperplasia (Fig.1-c), respectively.

Pseudohyphae or hyphae penetrated vertically into the keratinized layer or upper layer of the prickle layer of papilloma with candidiasis (Fig.1-b).

Neutrophilic infiltration was observed in the connective tissue and was invaded in the epithelial tissue (Fig.1-d). PAS reaction revealed clearly hyphal invasion (inset in Fig.1-b and Fig.1-d).

Figure 2

Longitudinal section of *Candidal* hyphe (HL) was existed within the oral epithelium (E). Cross section of *Candidal* hyphe (HC) internalized into the epithelial cell inside a cytoplasmic vacuole (V). The cell covered with plasma membrane (PM) is surrounded by thick cell wall (CW). (Fig.2-1:x5,000).

Candial hypha containing mitochondrias (M) and a nuclear (N) had invaded along an intercellular bridge was observed. Desmosomal cell junction (D) of the epithelial cells (E) and dense tonofilament (TF) were arranged irregularly in the host cytoplasm. Destruction of desmosomal cell junction (arrow) and deficit of tonofilament (arrowhead) were also observed at the tip of the hypha (Fig.2-2:x10,000). “C” means candidiasis in the figures.

Figure 3

In the without candidiasis group, a strong positive reaction was seen across the whole of the prickle layer, which presented a clearly scalar appearance (Figs.3-a,c). In the candidiasis group, weaker expression was seen in the prickle layer (Figs.3-b (1),d(1)). Strong positive E-cadherin reaction was seen along the insertion sites of the hyphae (Figs.1-b(2),d(2), arrowhead). “C” means candidiasis in the figures.

Figure 4

CK13 was negative in the basal layer of papilloma and verrucous hyperplasia without candidiasis (Figs.4-a1,c1). In papilloma and verrucous hyperplasia with candidiasis, weakly positive in the basal layer and strongly positive in the whole

prickle layer. (Figs.4-b1,d1).

EGFR was almost negative in without candidiasis groups (Figs.4-a2,b2,c2,d2). Weakly positive EGFR reactions were observed in the upper layer of the prickle layer in papilloma and verrucous hyperplasia with candidiasis (Figs.4-b2,d2).

Higher values for Ki-67 positivity were found in both the basal layer and the prickle layer of the candidiasis group (Figs.4-b3,d3) than the without candidiasis group (Figs.4-a3,c3). “C” means candidiasis in the figures.

Figure 5

NE and CD68 were almost completely negative the without candidiasis group (Figs.5-a1,c1,a2,c2). In papilloma and verrucous hyperplasia with candidiasis, strongly positive reactions were seen on the prickle layer and connective tissue (Figs.5-b1,d1,b2,d2).

COX-2 was negative in the without candidiasis group (Figs.5-a3,c3). In the candidiasis group, COX-2 was positive in all layers in papilloma (Fig.5-b3), and mix of strongly and weakly positive reaction was seen in verrucous hyperplasia (Fig.5-d3).

In the investigation of new blood vessels using CD105 (Figs.5-a4,b4,c4,d4),

numerous enlarged blood vessels were found in the connective tissue immediately below the basal membrane in papilloma (Fig.5-b4) and verrucous hyperplasia with candidiasis (Fig.5-d4). “C” means candidiasis in the figures.

Table 1 Subjects of immunohistochemical study

Pathological Diagnosis (Average age ±S.D.)	Age	Sex	Location
Normal oral mucosa	22	F	Gingiva
	32	F	Gingiva
	11	M	Tongue
	26	F	Gingiva
	39	M	Gingiva
Papilloma without Candidiasis (46.3±16.5)	50	M	Palate
	50	M	Palate
	25	F	Gingiva
	25	F	Gingiva
	54	F	Tongue
	42	F	Tongue
	71	M	Tongue
	54	F	Tongue
Papilloma with Candidiasis (54.0±20.0)	55	F	Lip
	59	F	Palate
	28	F	Palate
	64	F	Tongue
	84	M	Tongue
	69	F	Tongue
	25	F	Tongue
	48	M	Tongue
Verrucous hyperplasia without Candidiasis (58.3±14.8)	40	M	Palate
	59	F	Palate
	72	F	Gingiva
	70	M	Gingiva
	66	F	Gingiva
	66	F	Gingiva
	35	M	Tongue
Verrucous hyperplasia with Candidiasis (69.2±2.7)	68	M	Palate
	71	M	Palate
	65	M	Tongue
	71	F	Tongue
	71	M	Tongue

M : Male
F : Female
S.D. : Standard Deviation

Table 2 Antibodies and retrieval methods used in the present study

Parpose	Antibody	Clone	Dilution	Company	Retrieval methods
1) Cell adhesion					
	E-Cadherin	NHC-38	1:100	DakoCytomation	Heat 121℃, 5min
2) Cytokeratin					
	Cytokeratin (CK13)	DE-K13	1: 50	DakoCytomation	Microwave, 15min
3) Proliferative factor					
	EGFR, Wild-Type	Dak-H1-WT	1:200	DakoCytomation	Heat 121℃, 5min
	Ki-67 Antigen	MIB-1	1:100	DakoCytomation	Heat 121℃, 5min
4) Inflammatory response					
	Neutrophil Elastase (NE)	NP-57	1:100	DakoCytomation	-
	CD68	KP1	1:100	DakoCytomation	Proteinase K, 5min
	COX-2	CX-294	1:100	DakoCytomation	Microwave, 15min
	CD105	SH6h	1: 10	DakoCytomation	Proteinase K, 5min

Table 3 Results of histopathological and immunohistochemical stainings

		H.E.		E-cadherin				CK13			PI (Ki-67)		EGFR		
			MI		Prickle				Prickle		Basal	Prickle		Prickle	
Case	No.	Inflammation	Ave.±SD	Basal	Lower	Upper	Keratinized	Basal	Lower	Upper	Ave.±SD	Ave.±SD	Basal	Lower	Upper
Normal oral mucosa	5	none to slightly	0.2±0.5	＋～±	＋	±～＋	－	－	±～＋	＋	4.7±0.5	0.1±0.1	－	－	－
Papilloma without candidiasis	8	none to slightly	0.6±0.2	＋	＋＋	＋＋～＋	－	－	＋～＋＋	＋～＋＋	26.1±10.9	0.3±0.5	－	－～±	－～±
Papilloma with candidiasis	8	mild to moderate	1.4±0.0 *	＋～±	＋～±	±～－	＋	±	±～＋	＋～±	28.5±14.3	0.3±0.7	－	－～±	±～＋
Verrucous hyperplasia without candididasis	7	none to slightly	0.5±0.3	＋	＋＋	＋＋～＋	－	－	＋～＋＋	＋～＋＋	12.1±13.9 *	0.1±0.1 *	－	－～±	－～±
Verrucous hyperplasia with candidiasis	5	mild to moderate	0.7±0.4	＋～±	＋～±	±～－	＋＋	±	＋～＋＋	±～＋	32.7±14.4	0.7±2.2	－	－～±	±～＋

Basal : Basal cell layer
Prickle : Prickle cell layer
MI : Mitosis index
PI : Proliferative index
E-cadherin, CK13, and EGFR : ＋＋ (strong positive), ＋ (moderate positive), ± (weak positive), － (negative)
＊ : Chi-square test, $p < 0.001$

Table 4 Results of immunohistochemical stainings for inflammatory response

		NE			CD68	COX－2			CD105	
		Epithelium		Connective tissue			Prickle		Length	Area
Case	No.	Surface	Prickle			Basal	Lower	Upper	Ave.±SD	Ave.±SD
Normal oral mucosa	5	－	－	－	－～±	－	－	－	89.33±7.1	9.4±2.2
Papilloma without candidiasis	8	－	－	－	－～±	－	－	－	170.8±2.8	9.3±4.8
Papilloma with candidiasis	8	＋＋	＋	＋	＋＋	＋	＋	＋	86.0±1.4	**159.7±180.8
Verrucous hyperplasia without candididasis	7	－	－	－	－～±	－	－	－	409.4±8.2	13.6±9.6
Verrucous hyperplasia with candidiasis	5	＋＋	＋	＋	＋＋	－～±	＋＋～±	＋＋～±	**119.7±1.7	96.5±159.3

Basal : Basal cell layer
Prickle : Prickle cell layer
COX－2 : ＋＋ (strong positive), ＋ (moderate positive), ± (weak positive), － (negative)
NE, CD68 : ＋＋ (high density), ＋ (large number), ± (small number), － (nothing)
Distance : Average of distance between basal membrane and vessels
Area : Average of vessel's area
＊＊ : Mann－Whitney U-test, $p < 0.001$