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Two-phase appearance of oral epithelial dysplasia resulting from focal proliferation of parabasal cells and apoptosis of prickle cells

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BACKGROUND: One of the histologic characteristics of epithelial dysplasias of the oral mucosa is droplet-shaped rete processes resulting from a solid proliferation of basaloid cells. These basaloid cells are suddenly changed into an overlay of parakeratotic cells. However, it is unknown how this characteristic two-phase appearance is generated.

METHODS: Formalin-fixed paraffin sections of the oral mucosal specimens with normal, hyperplastic, dysplastic epithelia and squamous cell carcinomas were examined for apoptosis by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method and for lymphoid cells by immunohistochemistry.

RESULTS: Apoptotic cells were only located in the keratinized layer of normal/hyperplastic epithelia. However, in epithelial dysplasias, apoptotic cells were scattered in the middle or even in the lower parts of the epithelial layer with frequent vacuolation changes of epithelial cells. Within the epithelial layer of dysplasias, there were increased number of lymphocytes, which were immunopositive for CD45RO, CD8, and CD57- and CD68-immunopositive (+), S-100 protein-positive and major histocompatibility complex (MHC) class II-positive monocytic lineages. They increased in number with the severity of dysplastic degrees, and they were often located in the vicinity of apoptotic epithelial cells, but decreased in carcinomas *in situ* and invasive carcinomas, which contained fewer numbers of apoptotic figures.

CONCLUSION: The findings indicate that intraepithelial infiltrations of both cytotoxic T cells and natural killer cells are closely related to the apoptotic phenomena of prickle cells, which may result in the characteristic 'two-phase appearance' of epithelial dysplasia.

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Introduction

It has been generally accepted that some of the oral squamous cell carcinomas develop through their precancerous steps of squamous epithelial dysplasia (1–3). Histopathologically, epithelial dysplasia has been conventionally classified into three grades: mild, moderate, and severe, although the relationship of these grades to the subsequent development of cancer has not been fully clarified. The WHO Histological Typing of Cancer and Precancer of the Oral Mucosa, second edition (1997) proposed 13 characteristic histologic changes that may occur in epithelial dysplasia (1). These include the following: loss of polarity of the basal cell (no. 1), the presence of more than one layer having a basaloid appearance (no. 2), drop-shaped rete-ridges (no. 4), irregular epithelial stratification (no. 5), and the presence of mitotic figures in the superficial half of the epithelium (no. 8). All of these may be caused by a solid proliferation of basaloid cells, which are most primitive in the epithelial layer and are not differentiated to basal cells or prickle cells. When such a monotonous proliferation of parabasal cells is pronounced, the transition from parabasal cell masses to the parakeratinized layer is greatly contrasted. In this study, we tentatively refer to this characteristic appearance of epithelial dysplasia as a 'two-phase appearance'. However, it is unknown how this contrastive transition occurs in these particular histologic architectures of epithelial dysplasia.

In our daily work performing surgical pathology services, we have noticed that the characteristic two-phase appearance of epithelial dysplasia is associated with vacuolated changes of epithelial cells or with lymphoid cell infiltrations including Langerhans cells. Based on this experience, we speculated a possibility of apoptotic processes among epithelial cells in epithelial dysplasia. Apoptosis in the oral mucosal epithelium has

been investigated by many investigators. However, they have mainly paid attention to the increasing numbers of apoptotic cells during the developmental steps of epithelial dysplasia from normal mucosa (4–10) or the apoptosis-associated protein in cancerization (11), and inhibition of apoptosis, which causes carcinogenesis (5, 9). There have been no studies on how apoptotic processes occur in the prickle layer of oral mucosal dysplasia. The presence of lymphoid cells including natural killer (NK) cells or cytotoxic T cells has been clarified within the epithelial layer of the squamous cell carcinoma of head and neck (12), lichen planus (13, 14), oral submucous fibrosis (15) or leukoplakia (16). In *in vitro* studies, NK cells or cytotoxic T cells have been shown to cause DNA breakdown in target cells by several apoptotic pathways (17–20). Apoptotic cells are then scavenging by macrophages or neighboring sibling cells (21). We therefore, consider that apoptosis in the prickle layer of oral mucosal dysplasia is closely related to increases of intraepithelial lymphoid cells including macrophages.

In the present study, we intended to determine the possibility of our hypothesis mentioned above by performing terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) techniques for distribution of apoptotic cells in epithelial dysplasia and by performing immunohistochemistry for the intraepithelial distribution modes of lymphoid cells to identify the T-cell subpopulation and NK cells as well as antigen-presenting cells, such as Langerhans cells.

Materials and methods

Materials

A total of 110 biopsy or surgical specimens from the oral mucosa were selected from the surgical pathology files in the Division of Oral Pathology, Niigata University Graduate School of Medical and Dental Sciences during a 7-year period from 1995 to 2001 after critical reviewing of hematoxylin and eosin (H & E) stained sections. These consisted of 15 cases of normal epithelium, 20 of hyperplasia, 45 of epithelial dysplasia (mild, 20; moderate, 15; severe, 10), and 30 of squamous cell carcinomas (well-differentiated, 20; poorly-differentiated, 10). The intraoral sites of the specimens taken from were as follows: gingiva, 20; tongue, 30; hard palate, 15; buccal mucosa, 20; soft palate, five; oral floor, 20. Three oral pathologists with the Japanese Society of Pathology board certification screened the specimens, when the diagnoses of grading of epithelial dysplasias are not identical the case will be re-evaluated together. All of the specimens were routinely fixed in 10% formalin and embedded in paraffin. Serial 5 μm sections were cut from paraffin blocks. One set of the sections was stained with H & E and was used for re-evaluation of histologic diagnosis, and the other sets were used for TUNEL staining for apoptosis as well as immunohistochemistry for lymphoid cells.

Antibodies

Mouse monoclonal antibodies against CD68 (PG-M1, IgG_3) (22), CD45RO (UCHL-1, IgG_{2a}) (23), CD8 (C8/144B, IgG_1) (24), and major histocompatibility complex (MHC) class II (human leukocyte antigen, HLA-DR) (CR3/43, IgG_1) (25) were obtained from Dako Ltd. (Glostrup, Denmark; diluted at 1:100, 1:75, 1:50, 1:75 respectively). A mouse monoclonal antibody against CD4 (1F6, IgG_1) (26) was purchased from Novocastra Laboratories Ltd. (Newcastle, UK, 1:10), CD57 (NK-1, IgM) (27) was purchased from Zymed Laboratories, Inc. (South San Francisco, CA, USA). Rabbit polyclonal antibodies against S-100 protein (28) were also purchased from Dako (1:500). For control experiments, the primary antibodies were replaced with pre-immune rabbit IgG (Dako) or mouse $IgG_1/IgG_3/IgG_{2a}$/IgM (Dako) according to the primary antibody classes.

Immunohistochemistry

Immunohistochemical experiments were performed by using Envision peroxidase systems for rabbit and mouse antibodies (Dako). After deparaffinization and dehydration, sections were washed in 0.01 M phosphate-buffered saline (PBS). Stainings for S-100 protein and CD68, sections were digested with 0.2% (w/v) trypsin (type II; Sigma Chemical Co., St Louis, MO, USA) in 0.05 M Tris-HCl (pH 7.6) containing 1% $CaCl_2$ for 30 min at 37°C to restore the antigenic sites. For CD8, CD4, CD56, and MHC class II, sections were autoclaved in 0.01 M citrate buffer (pH 6.0) for 15 min at 121°C and then kept standing for 20 min at room temperature. To block endogenous peroxidase activities, all the sections were quenched with 0.001% H_2O_2 in 100% methanol for 30 min at room temperature and rinsed with PBS-containing 0.5% skim milk and 0.05% triton X-100 (PBST). After rinsing in PBST, the sections were incubated in 5% skim milk in PBS-containing 0.05% triton X-100 for 1 h at 37°C to block non-specific protein bindings. The sections were then incubated with mouse monoclonal primary antibodies against CD68, CD45RO, CD8, CD4, CD57, and MHC class II, or with rabbit polyclonal antibodies against S-100 protein, for overnight at 4°C. After incubations with the primary antibodies, the sections were rinsed in PBST and then treated with polymer-immune complexes (EnVision+ peroxides, rabbit/mouse; Dako, 1:1) for 1 h at room temperature. The peroxidase reaction products were visualized by incubation with 0.02% 3,3'-diaminobenzidine (DAB; Dohjin Laboratories, Kumamoto, Japan) in 0.05 M Tris-HCl solution (pH 7.6) containing 0.005% H_2O_2. The sections were counterstained with hematoxylin.

In situ *detection of apoptosis*

In situ detection of apoptotic cells in paraffin section was performed by the TUNEL method using an *in situ* cell death detection kit (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, after deparaffinization and dehydration, sections were autoclaved and blocked for endogenous peroxidase activities and non-specific protein bindings in the same way as mentioned above. After rinsing in PBST, the sections were incubated with a TUNEL reaction mixture containing fluorescein-dUTP, dNTP, and TdT in 0.2 M potassium cacodylate,

0.025 M Tris-HCl, 0.001 M $CoCl_2$, 0.25 mg/ml bovine serum albumin (pH 6.6) for 1 h at 37°C according to the manufacturer's instructions. After washing with PBST, they were then incubated with antifluorescein antibodies for 30 min at room temperature. Reaction products were confirmed on a fluorescent microscope, and then were further visualized by incubation with DAB. The sections were counterstained with hematoxylin. For negative control experiments, sections were incubated with the TUNEL reaction mixture without TdT. In addition, competitive assays using unlabelled dUTP (Roche) in different ratios to fluorescent-dUTP as 1:1, 1:10, and 1:100 (29). For positive controls, sections were treated with DNase I (1 μg/ml) for 10 min at room temperature before incubation with the TUNEL reaction mixture.

Statistical analysis

The numbers of T lymphocyte subsets (CD45RO+ cells, CD8+, CD4+), CD57+ NK cells, and apoptotic cells in a square unit of 1 mm^2 were counted on a microscope equipped with a micrometer. Ten fields were randomly counted per section at ×100 magnification. One-way ANOVA was used for statistical comparison of cell numbers between each group by using the SPSS software program (SPSS Inc., Chicago, IL, USA).

Results

Recognition of epithelial dysplasia with two-phase appearance

The 45 samples of epithelial dysplasia were classified into three grades basically according to the WHO standard (1). In our study, however, when a solid proliferation of basaloid cells with a complete or partial loss of basal cell alignment was recognized in the lower half of the epithelial layer, we regarded it as a hallmark of the moderate grade. The basaloid cell proliferation some cases occurred focally (Fig. 1a) or continuously (Fig. 1b) within the epithelium, and they seemed to be primitive parabasal cells which were not yet differentiated into upper prickle cells or lower basal cells. This basaloid cell proliferation was frequently associated with vacuolar changes of epithelial cells in the upper layer (Fig. 1c), and basaloid cells were sometimes mixed with vacuolated cells (Fig. 1d). Since this middle zone with vacuolar cells, which should have corresponded to the prickle cell layer, was narrow and gave an

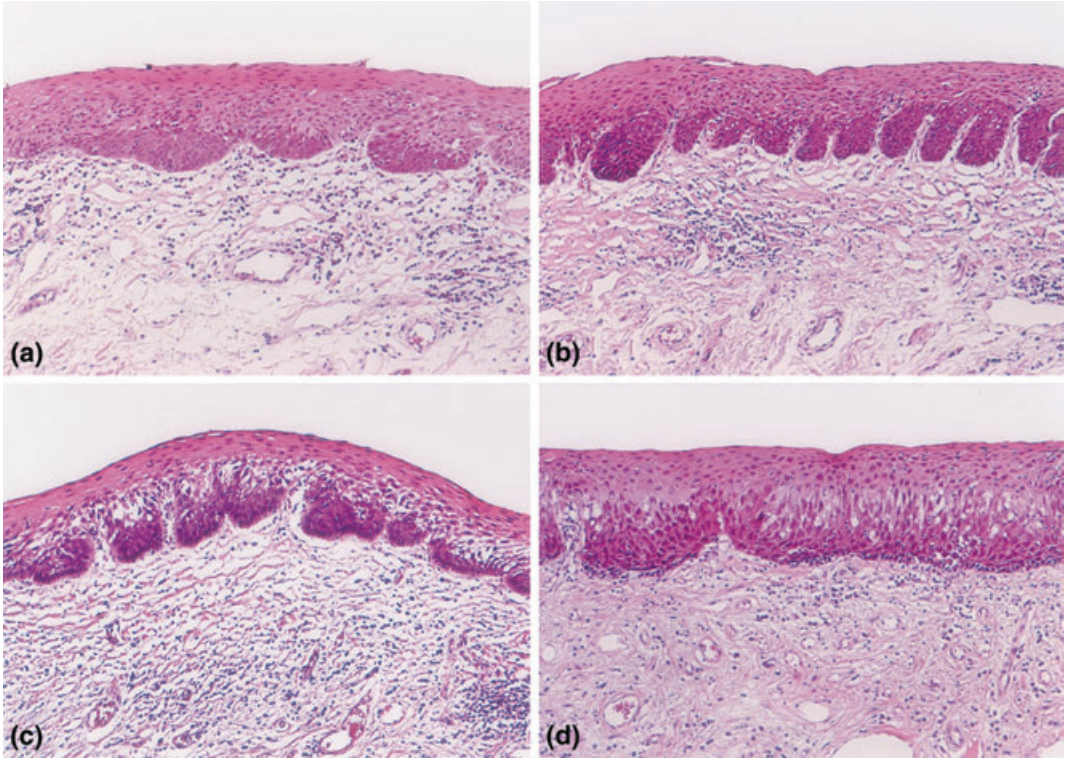

Figure 1 Epithelial dysplasia with two-phase appearance. (a) Focal proliferation of basaloid cells budding from the basal end of the epithelium into the lamina propriae; (b) continuous solid proliferation of basaloid cells in the lower half of the epithelium; (c) apparent increase of vacuolated cells and their association with infiltrations of lymphoid cells in the middle zone; (d) extension of basaloid cells toward the upper layer resulting in intermingling with vacuolated cells. Hematoxylin and eosin stain (H & E), ×125. Independent and smaller foci of parabasal cell proliferation (a) seemed to merge with each other and occupied the lower half of the epithelium (b). There was a definite contrast between the lower and upper layers. Epithelial dysplasia with such a sudden change in the epithelial cell feature was called epithelial dysplasia with a two-phase appearance. The interface zone containing vacuolated cells, which may have corresponded to the prickle cell layer, seemed to be compressed and were narrow due to sandwiching between the lower solid proliferation of basaloid cells and the upper keratinized cell layer (c). These epithelial dysplasia cases with a two-phase appearance were classified into the moderate degree (a–c). When the alignment of vacuolated cells in the middle zone disappeared, basaloid cells proliferated toward the upper layer and were intermingled with vacuolated cells (d).

impression of a sudden transition from the lower solid zone of basaloid cells to the upper keratinized layer, it was considered that the prickle cell layer was disappearing. We called this type of epithelia dysplasia the 'epithelial dysplasia with a two-phase appearance'. Such kind of epithelial dysplasia with the characteristic two-phase appearance was frequently found in the vicinity of foci of carcinoma *in situ* or squamous cell carcinoma with superficial spreads. In this study, carcinoma *in situ* was used as a synonym of severe dysplasia. A diagnosis of severe dysplasia/carcinoma *in situ* was not only applied for cases with a whole layer replacement with basaloid cells but also for those with an apparent tendency toward keratinization if a total loss of basal cell alignment was associated. Thus, we diagnosed lesions with two-phase appearance as moderate dysplasia.

In situ *detection of apoptosis*

In normal and hyperplastic epithelia (Fig. 2a), apoptotic cells, which were demonstrated by TUNEL, were located only in their surface keratinized layers (Fig. 2b). However, in epithelial dysplasia, moderate, with a two-phase appearance (Fig. 2c, e), a significant number of apoptotic cells were also found in the lower layers of the epithelium, in addition to the surface layer. The most conspicuous examples were those with apoptotic figures concentrated in the middle layer of the dysplastic epithelium with a two-phase appearance (Fig. 2d, f), as mentioned above. Apoptotic cells in the epithelial dysplasia were also scattered irregularly in the basal or parabasal layers (Fig. 2f), and such apoptotic figures in abnormal positions seemed to be associated with epithelial cells with vacuolation changes. TUNEL signals were not only localized in nuclei but occasionally in the cytoplasm. They increased in number with the severity of epithelial dysplasia up to moderate degrees. However, in severe dysplasia/carcinoma *in situ* and early invasive carcinoma, apoptotic cells decreased in number and tended to be located more irregularly (Fig. 2g). When lower layers were totally occupied with basaloid cell proliferation, apoptotic cells were restricted to the surface layer (Fig. 2h). There were only a few apoptotic cells in invasive carcinoma cell nests (not shown). Quantitatively, the numbers of apoptotic cells in mild ($9.9 \pm 6.0/mm^2$) and moderate (13.4 ± 7.2) epithelial dysplasia were significantly higher than those in normal/hyperplastic epithelia (2.5 ± 4.8; $P < 0.05$) or than those in severe dysplasia/carcinoma *in situ* (2.5 ± 3.8; $P < 0.05$), and carcinoma (1.3 ± 1.2; $P < 0.05$) (Fig. 3). In control experiments, positive stainings were obtained in all of the nuclei in sections when they were pretreated with DNase I, and not when they were incubated with incomplete TUNEL solutions or when they were incubated with increased concentrations of unlabeled TdT in the TUNEL mixtures.

Immunohistochemistry for infiltrating cells

As moderate epithelial dysplasia with a two-phase appearance was shown to have resulted from the presence of concentrated apoptotic figures in the middle layer of the epithelium, it was interesting to investigate the composition of intraepithelial cells infiltrating against apoptotic cells in order to understand how such unusual apoptoses were induced and how apoptotic cells were scavenged.

At first, intraepithelial lymphocytes were immunohistochemically examined in terms of their subclasses. There was no discernible number of B cells in the epithelial layer of the oral mucosa ranging from normal to dysplasia. However, there was a significant degree of T-cell infiltration in the same spectra of the epithelium. The presence of CD45RO+ T cells was recognized within normal and hyperplastic epithelia. They were located singularly in the lower half of the epithelial layer (Fig. 4a). In epithelial dysplasia, there were a prominent number of T cells not only in the lamina propriae but also in the epithelial layer (Fig. 4b). However, most of the CD45RO+ cells were located in the lamina propriae, and a small number of them were within the epithelial layer. This gave an impression that the basement membrane of the epithelium was resistant to the lymphocytic penetration. T cells increased in number with the severity of epithelial dysplasia up to severe dysplasia/carcinoma *in situ*, and then decreased in the carcinomas. Figure 5a shows the comparison of intraepithelial T-lymphocyte populations among these different epithelial conditions. The difference was statistically significant between normal/hyperplasia and moderate and severe dysplasia/carcinoma *in situ* ($P < 0.05$, respectively).

Among the intraepithelial T cells, CD8+ cells represented an increase in number with the severity of epithelial dysplasia (Fig. 4c). In contrast, CD4+ cells did not change in number with the severity of dysplasia (Figs 4d and 5b). The ratio of the number of cells between CD8+ and CD4+ in the epithelial layer was 6:1 in normal/hyperplastic epithelia, and it was extended up to 11:1 in severe dysplasia, which indicated that CD8+ cells were responsible for the increase of intraepithelial lymphocytes. CD57+ cells, which are regarded as NK cells, also increased in number with the severity of epithelial dysplasia up to moderate dysplasia (Fig. 4e), but they decreased in severe dysplasia/carcinoma *in situ* and in invasive carcinoma (Fig. 6). There were statistically significant differences between normal/hyperplasia and moderate dysplasia ($P < 0.05$) and between invasive carcinoma and moderate dysplasia ($P < 0.05$) (Fig. 6).

As for the monocytic cell populations, immunohistochemistry for CD68 (Fig. 4f), S-100 protein (Fig. 4g), and HLA-DR (Fig. 4h) revealed the presence of macrophages, dendritic cells, or Langerhans cells within the epithelium, although most of the immunopositive cells were found more in the lamina propriae than in the epithelium. The numbers of intraepithelial S-100 protein-positive and HLA-DR+ cells were more than CD68+ cells. The S-100 protein-positive, and HLA-DR+ cells were more densely localized in the basal layer but extended in the middle layer of dysplastic epithelia, especially in the vicinity of apoptotic epithelial cells or vacuolated cells and CD8+ or CD57+ cells. In addition, these monocyte-lineage cells obviously

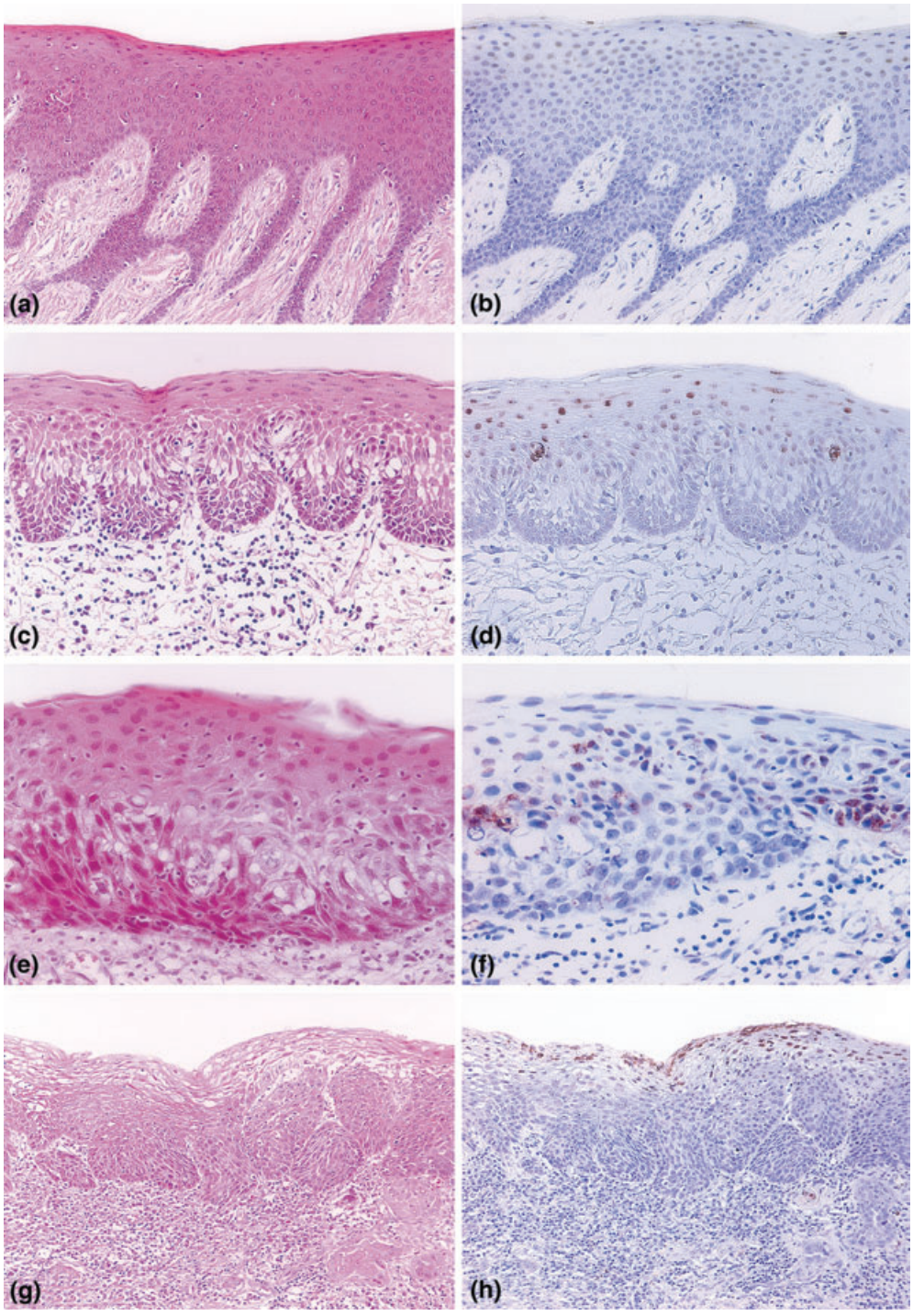

Figure 2 Demonstration of apoptotic cells in the oral mucosal epithelia. (a and b) Normal/hyperplastic epithelium, ×200; (c and d) epithelial dysplasia, moderate, ×200; (e and f) severe dysplasia carcinoma *in situ* ×200; (g and h) early invasive carcinoma; (a, c, e and g) hematoxylin and eosin stain (H & E); (b, d, f and g) terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) immunoperoxidase, hematoxylin counterstain. In normal/hyperplastic epithelia (a), TUNEL-positive cells were occasionally found in the surface keratinized layer (b). However, TUNEL-positive cells increased in number in epithelial dysplasia up to a moderate degree (d). In moderate epithelial dysplasia, vacuolated cells increased in the middle layer and there was a definite contrast between the lower layer of basaloid cell proliferation and the upper keratinized layer (c). Epithelial dysplasia with a two-phase appearance seemed to have resulted from apoptotic disappearance of prickle cells. With severity of epithelial dysplasia (e), apoptotic cells decreased in number and were located in lower layers (f). In severe dysplasia carcinoma *in situ* with massive proliferation of basaloid cells or early invasive carcinoma (g), apoptotic figures were restricted to only the surface layer (h).

increased in number in the mild degree of epithelial dysplasia and decreased with the severity of epithelial dysplasia up to invasive carcinoma (Fig. 7). There were statistically significant differences only between mild dysplasia and normal/hyperplasia and carcinoma ($P < 0.05$), although the difference of their population

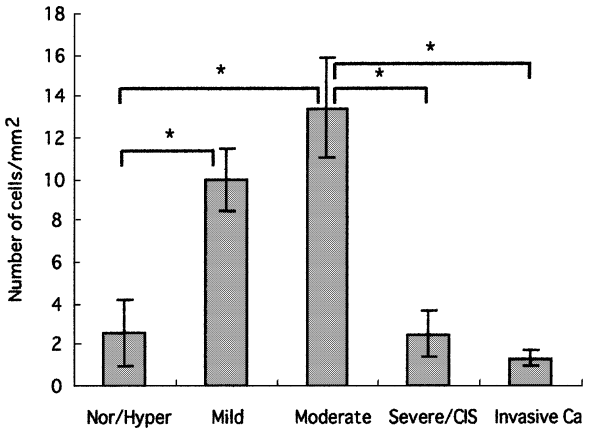

Figure 3 Quantification of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL)-positive cells in the oral mucosal epithelia from normal/hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia/carcinoma *in situ*, and invasive carcinoma. TUNEL-positive cells were counted in 10 random fields of 1 mm^2 units within the epithelial layer on a microscope equipped with a micrometer at ×100 magnification. The values in mild and moderate dysplasia had a statistically significant difference (*$P < 0.05$, one-way ANOVA) in comparison with those of other lesions.

in other epithelial conditions was not statistically significant. In epithelial dysplasia, HLA-DR was occasionally immunolocalized in the epithelial cells from the basal to the upper layers (Fig. 4h), while there was no definite immunopositivity for HLA-DR in normal/hyperplastic epithelia.

Discussion

The present results demonstrated distinct modes of localization of apoptotic cells between normal/hyperplastic and dysplastic epithelia of the oral mucosa. The most striking results were that apoptotic cells were accumulated in the lower to middle layers of dysplastic epithelia, whereas they decreased in number in severe dysplasia/carcinoma *in situ* or in invasive carcinoma. The presence of apoptotic cells in the surface keratinized layer of normal oral epithelium has been documented by using the TUNEL method (9–11), suggesting that desquamation of keratinized cells is synonymous with the apoptotic terminus of squamous epithelial cells. In contrast to these results, Kohno et al. (5) reported the presence of apoptotic figures only in the suprabasal layer of normal epithelium of hamster oral mucosa. Birchall et al. reported similar results from human oral epithelium (8). Although there have been several investigations on apoptosis in human oral mucosal epithelium from normal, dysplastic, and carcinomatous conditions, apoptotic cell locations within the epithelium were not always described (4, 6, 7). As reported by Loro et al. in oral epithelium (29) and rat vaginal epithelium (30) TUNEL method may detect cell deaths not only by apoptosis but also through terminal differentiation toward keratinization (29). Thus, it still seems to be controversial as to where apoptosis occurs in normal squamous epithelium. Although it is difficult to explain the reason for the discrepancy among the reports, it should be emphasized that our TUNEL method by using fluorescein-labeled dUTP and anti-fluorescein antibodies has been shown to be more sensitive than other TUNEL methods and equally accurate in comparison with other apoptosis detection methods (31).

In the present study, we could demonstrate that apoptotic cells increased in number with the severity of epithelial dysplasia up to moderate degrees and then decreased from severe dysplasia/carcinoma *in situ* to invasive carcinoma. The increase of apoptotic cells in epithelial dysplasia has already been reported by Kohno et al. (5) and Birchall et al. (7), although severe dysplasia/carcinoma *in situ* were most highly TUNEL-labeled in the latter study and severe dysplasia/carcinoma *in situ* were not separately evaluated from dysplasias of lesser degree in the former. In contrast to the above investigations in which apoptotic figures decreased in number in malignant conditions, Macluskey et al. (4) showed increasing tendencies of apoptosis toward the progression of oral squamous cell carcinoma. Similar results were obtained both in human tissues and experimental carcinogenesis in rat and hamster oral mucosa (5, 6, 9). Thus, it is also controversial in terms of the frequency of apoptotic figures in the process of cancerization of the oral mucosal epithelium from normal to invasive carcinoma through dysplasia.

In spite of these discrepancies, what we would like to emphasize from the present study is the accumulation of apoptotic cells in the middle layer of dysplastic epithelia, especially those with the characteristic two-phase appearance. Since there has been no documentation on such a particular appearance of epithelial dysplasia in the literature, we would like to repeat here again the use of the term 'two-phase appearance'. Epithelial dysplasia with a two-phase appearance was frequently found in the vicinity of carcinoma *in situ* and in early invasive stages of squamous cell carcinoma in our series. This peculiar appearance seems to be due to a focal proliferation of basaloid cells with disappearance of basal cell alignments, which may compress the upper prickle cell layer from the lower side. Between the lower basophilic layer which is composed of basaloid cells and the upper eosinophilic layer composed of parakeratinized cells, there seems to be no apparent zone of prickle cells. We propose to call this contrastive interfacing of the two layers with different characteristics a 'two-phase appearance'. Based on the present TUNEL results in the dysplasia cases with two-phase appearances, it is highly suggested that this particular appearance is due to apoptosis of prickle cells. It is unknown from the present study how apoptosis is induced in this interface position. However, mechanical stress by sandwiching seems to be a candidate for this, because apoptotic pathways from mechanical stress have been demonstrated in epithelial cells in pulmonary (32), liver (33), and kidney (34) lesions or in muscle (35) and bone (36) cells. In epithelial cells, keratin filaments have been considered as a resistant device of mechanical stress-induced apoptosis (37), and mechanical stretching signals seem to be mediated by integrins (35).

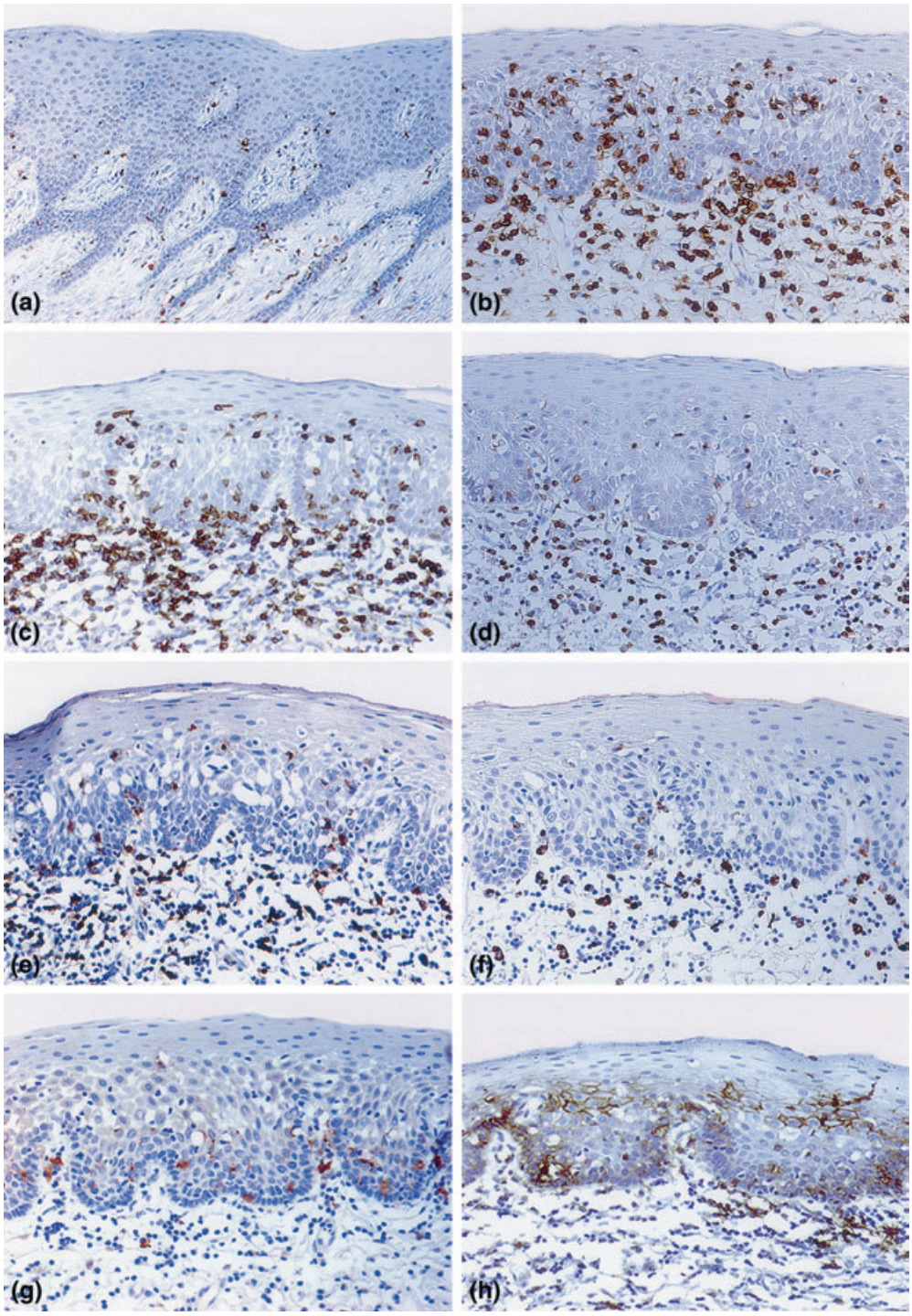

Figure 4 Immunohistochemical localization of lymphoid cells in moderate oral mucosal epithelia. (a) Normal/hyperplastic epithelium, ×200; (b–h) epithelial dysplasia, moderate, ×200. Immunoperoxidase for CD45RO (a and b), CD8 (c), CD4 (d), CD57 (e), CD68 (f), S-100 protein (g) and major histocompatibility complex (MHC) class II (h). Hematoxylin counterstain, ×200, Only CD8-positive cells were found within the epithelial layer of normal/hyperplastic epithelia, but they increased in number in epithelial dysplasia (c), which seemed to represent most of the intraepithelial T cells revealed by CD45RO immunohistochemistry (b). In contrast, CD4+ T cells within the epithelial layer were minimum but plentiful in the lamina propriae (d). CD57+ natural killer (NK) cells showed a similar distribution to CD8+ T cells. Within the epithelial layer, T cells and NK cells were both located in the vicinity of vacuolated cells (apoptotic cells). CD68+, S-100 protein-positive, and MHC class II-positive cells were similarly distributed within the epithelial layer as well as in the lamina propriae (f–h). Within the epithelial layer, they were also located in the vicinity of vacuolated cells. In addition to macrophage lineage cells and lymphocytes, parabasal cells in the lower half of the epithelium occasionally expressed MHC class II (h).

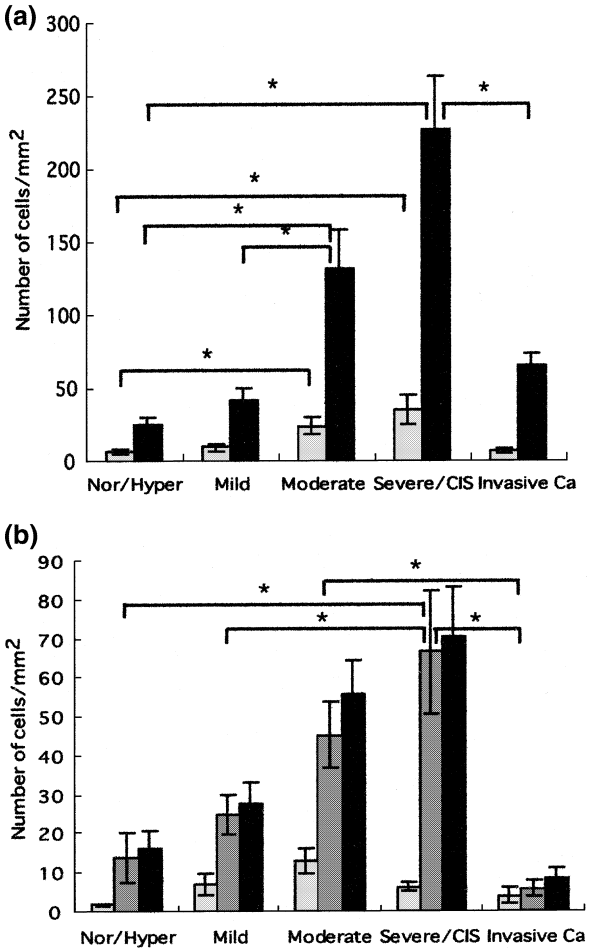

Figure 5 Quantification of T-cell infiltrations in the oral mucosal epithelia from normal/hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia/carcinoma *in situ*, and invasive carcinoma. Immunopositive cells were counted in 10 random fields of 1 mm^2 units within the epithelial layer or lamina propriae on a microscope equipped with a micrometer at ×100 magnification. (a) Comparison of CD45RO+ T-cell number between the epithelial layer and lamina propriae, □ epithelial layer, ■ lamina propriae. (b) Comparison of intraepithelial T-cell number between CD4+ and CD8+ cells. □ CD4, ■ CD8, ■ total T. Both intraepithelial and subepithelial T cells increased in number with the severity of epithelial dysplasia and decreased in invasive carcinoma, although those in the lamina propriae increased dramatically in moderate and severe dysplasia (a). Statistically, the increases of both intraepithelial and subepithelial T cells in moderate and severe dysplasia/CIS were significant in comparison with those from other lesions ($P < 0.05$). Most of the intraepithelial T cells were CD8+ cells (b).

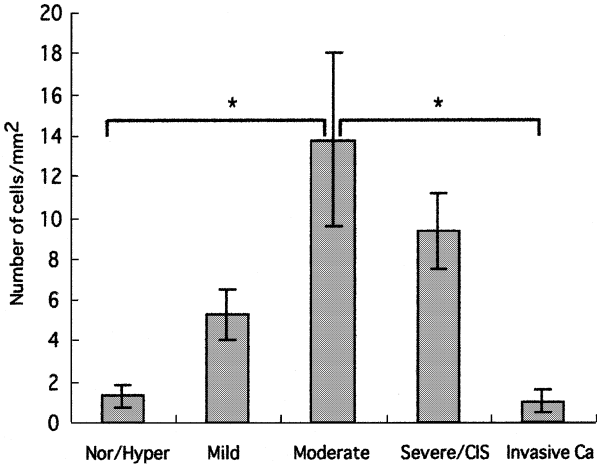

Figure 6 Quantification of CD57+ natural killer (NK) cell infiltrations in the oral mucosal epithelia from normal/hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia/carcinoma *in situ*, and invasive carcinoma. Immunopositive cells were counted in 10 random fields of 1 mm^2 units within the epithelial layer on a microscope equipped with a micrometer at ×100 magnification. CD57+ cells increased in number with the severity of epithelial dysplasia up to a moderate degree but decreased thereafter in severe dysplasia/carcinoma *in situ* and invasive carcinoma. ($*P < 0.05$)

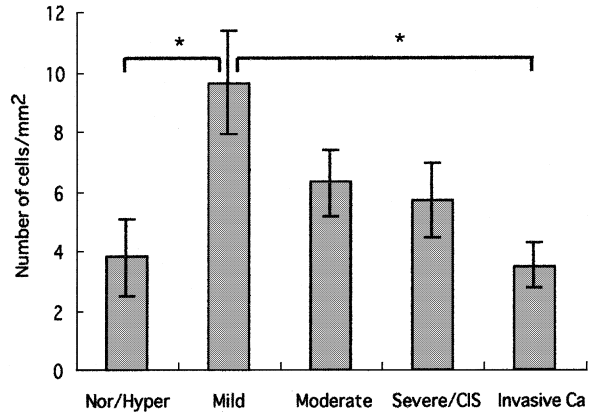

Figure 7 Quantification of S-100 protein-positive cell infiltrations in the oral mucosal epithelia from normal/hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia/carcinoma *in situ*, and invasive carcinoma. Immunopositive cells were counted in 10 random fields of 1 mm^2 units within the epithelial layer on a microscope equipped with a micrometer at ×100 magnification. S-100 protein-positive dendritic cells increased in number most in mild dysplasia then decreased in more advanced dysplasias, carcinoma *in situ*, and invasive carcinoma. Statistically, the value for mild dysplasia was significantly higher than that for normal/hyperplastic epithelia and invasive carcinoma. ($*P < 0.05$)

What is the fate of the epithelial dysplasia with a two-phase appearance? From our observation in this study that this particular form of epithelial dysplasia is frequently observed in the vicinity of severe dysplasia/carcinoma *in situ*, it is suggested that the basaloid cells proliferate focally in the lower layer of the epithelium and then replace the whole layer to become severe dysplasia/carcinoma *in situ*. After that, they may be differentiated again toward keratinization (38). In this step, apoptotic figures are expected to decrease in number from the middle layer, and instead apoptosis may be induced by a crowding condition in the lower layer of severe dysplasia/carcinoma *in situ*, which was observed in the present study as well as in the previous studies (5, 9).

When compared with epithelial dysplasia and carcinoma *in situ*, invasive carcinoma had obviously fewer apoptotic figures in the present study, and this was basically consistent with the previous studies (2, 7). Loro et al. (11) reported an increased number of apoptotic figures in carcinomas, which is a finding completely opposite of ours. However, they only compared those between normal epithelium and carcinoma and did not

examine dysplasia or carcinoma *in situ*. Since invasive carcinoma cells are more potent in proliferation, the decrease in the number of apoptotic cells in invasive carcinomas could be explained by the antiapoptotic mechanisms in a malignant cell proliferation (39).

The two-phase dysplasia was also analyzed from the aspect of lymphoid cell reaction in the present study. Our observation that CD8+ cells were more commonly located than CD4+ cells within the epithelium and vice versa in the lamina propriae in normal oral mucosa is consistent with the previous observations (15). However, there have been no accurate reports on intraepithelial distribution T-cell subsets in oral mucosa in the literature. Our present result showed immunohistochemical demonstration of T-cell subset distribution in normal and dysplastic epithelia of human oral mucosa for the first time. As the decrease of CD4/CD8 ratio was parallel with the increase of CD57+ NK cells and of severity of epithelial dysplasia, CD8+ cells and NK cells represent the increase of intraepithelial lymphocytes in dysplastic conditions. In addition, the co-localization of TUNEL-positive apoptotic epithelial cells and CD8+ or CD57+ cells may indicate that intraepithelial lymphocytes in epithelial dysplasia are involved in the apoptotic events of epithelial cells. Since TUNEL-positive cells were also co-localized with CD68+/S-100+/MHC class II-positive cells, which increased in number with the severity of epithelial dysplasia, it is highly suggested that apoptotic epithelial cells are engulfed in part by intraepithelial macrophages and mediated by dendritic cells or Langerhans cells, and but Travaglione et al. (40), also demonstrated neighboring epithelial cells could scavenged apoptotic cells. This result is quite reasonable with the present immunologic understanding on the apoptotic pathway induced by CD8+ T cells and CD57+ NK cells, in which exocytoses of fragmentin (18) and cytolysin (19) activate Fas ligand or perforin (20) and granzymes (21), resulting in DNA breakdown.

In addition to the dendritic cells both in the epithelium and in the lamina propriae, MHC class II was strongly expressed in dysplastic epithelial cells located in the lower epithelial layers in the present study. The expression of MHC class II has been already reported in epithelial cells in the fetal skin, kidney, and small intestines during the initiation step of immune responses (41) as well as in oral squamous cell carcinomas (42, 43). As the epithelial expression of MHC class II is known to be controlled by interferon-γ stimulations (44), dysplastic epithelial cells or carcinoma cells may be induced to express it when they are exposed to lymphoid cells which come into the epithelial layer after breaking through the basement membranes (13, 45).

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