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Original

Localization of bromodeoxyuridine-incorporating, p63- and $p75^{NGFR}$- expressing cells in the human gingival epithelium

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Abstract: The objective of this study was to find the sites with proliferative activity in the human gingival epithelium, where stem cells are likely to exist. Gingival tissues were excised from 16 adult patients and immunohistochemically examined for the presence of bromodeoxyuridine (BrdU)-incorporating, p63- and low affinity nerve growth factor receptor ($p75^{NGFR}$)-expressing cells. BrdU-incorporating cells were rarely present in the junctional epithelium. The number of BrdU-incorporating cells in the sulcular and oral gingival epithelia was significantly higher than that found in the junctional epithelium (ANOVA, $P < 0.01$). A considerable number of p63-positive nuclei were detected in the basal layer to lower spinous layers in the sulcular and oral gingival epithelia, but only few p63-positive cells were present in the junctional epithelium. $p75^{NGFR}$-positive cells were exclusively located in the basal layer in the sulcular and oral gingival epithelia, and in limited basal area in the junctional epithelium neighboring the sulcular epithelium. In the oral gingival epithelium, intense immunostaining of BrdU, p63 and $p75^{NGFR}$ was correspondingly observed on the base and side of the rete ridges. These areas probably exhibit high proliferative activity owing to the presence of stem cells. (J. Oral Sci. 49, 287-291, 2007)

Keywords: oral mucosal epithelium; BrdU; p63; $p75^{NGFR}$; immunohistochemistry.

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Introduction

Many adult tissues contain tissue-specific stem cells. For example, in the epidermis, there are stem cells that divide and generate themselves and transient amplifying cells, and then terminally differentiate after a discrete number of cell divisions (1-3). Such renewal has been shown to be concentrated around highly ordered structures termed epidermal proliferative units (EPU), each generated by a single stem cell (4,5). Similar to that in the epidermis, there might be proliferative units for renewal of the oral mucosal epithelium, however it is yet to be reported.

Three types of anatomically distinct epithelia are associated with the dentogingival junction, namely the junctional, sulcular, and oral gingival epithelia (6). The keratinizing oral gingival epithelium lines the external surface, while the non-keratinizing sulcular epithelium lines the inner sulcus. The junctional epithelium, which collars the tooth, has cellular characteristics different from those of the other two epithelia, such as the types of keratin (6) and adhesion molecules (7). Identifying the area with high proliferative activity in the three gingival epithelia, where stem cells are believed to exist, would certainly help to develop beneficial regenerative therapy for periodontal disease.

The potential candidates for epidermal stem cell markers, such as 5'- bromodeoxyuridine (BrdU) label-retaining cells in hair follicles (4), the integrin family in foreskin epidermis (8), p63 in the human cornea and epidermis (2,4,9-12), and low affinity nerve growth factor receptor, $p75^{NGFR}$ (13,14), have been studied. Therefore, we examined whether BrdU-incorporating cells and expression of p63 and $p75^{NGFR}$ would serve as putative stem cell markers in oral mucosal epithelium by the immunohistochemical method.

Materials and Methods

Tissue samples

Samples of gingival tissues were obtained with informed consent from 16 adult patients (7 males and 9 females, mean age: 56.7 ± 6.5 years, range: 46-66), who had been suffering from chronic marginal periodontitis and had thus undergone therapeutic flap surgery at the Dental Hospital of Iwate Medical University. We received permission to perform these procedures from the ethical committee of Iwate Medical University. All patients had detailed clinical records and radiographs. Gingival tissue samples were obtained from various sites. The tissue specimens were selected based on two histological findings: presence of junctional, sulcular, and oral gingival epithelia; and few chronic inflammatory cells in the sub-epithelial connective tissue.

BrdU labeling and the immunohistochemical detection of BrdU, p63 and $p75^{NGFR}$

All samples were cut along the vertical axis of the tooth and some of them were labeled in minimum essential medium (MEM, GibcoBRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), which included 1.6 μM BrdU (Sigma, St. Louis, MO, USA) for 2 h at 37°C. After incubation, they were washed, fixed in 4% paraformaldehyde, and then embedded in paraffin. The specimens were cut to a thickness of 4 μm and then routinely deparaffinized. They were treated with 4N HCl for 20 min and then consecutively with 4% borax for 20 min. The other samples were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. Next, for immunostaining, the specimens were immersed in 0.3% H_2O_2 in methanol for 20 min to block any endogenous peroxidase. The specimens were then reacted with primary anti-BrdU (Bu20a, Dako, Carpinteria, CA, USA), anti-p63 (4A4, Lab Vision Co., Fremont, CA, USA), and anti-$p75^{NGFR}$ (NGFR 5, Dako) antibodies overnight in a humid chamber at 4°C. After being washed with PBS, the specimens were incubated with EnVision secondary antibody reagent (Dako). After washing, the sections were incubated with 3,3'-diaminobenzidine (Wako Pure Chemical Ind., Osaka, Japan) for 15 min at room temperature, and were counterstained with Mayer's hematoxylin. For evaluation of the immunolabeled tissues of BrdU, the gingival epithelium was histologically divided into three regions for the healthy gingiva; namely, the oral epithelium, sulcular epithelium, and junctional epithelium. The number of BrdU-positive cells per 1 mm of the basement membrane based on magnified light microphotography was determined. The numbers of such cells in each region of the gingival epithelium were statistically analyzed by the one-way analysis of variance test.

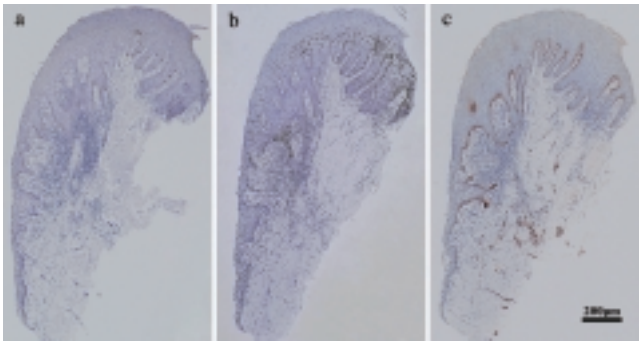

Fig. 1 Representative microscopic findings immunostained for BrdU (a), p63 (b) and $p75^{NGFR}$ (c) in human gingival epithelium.

Results

BrdU labeling

In most specimens, BrdU-incorporating cells were not detected in the junctional epithelium, but a small number of BrdU-positive nuclei were infrequently observed in the basal layer at the apical portions (Figs. 1a, 2a, 2b). In

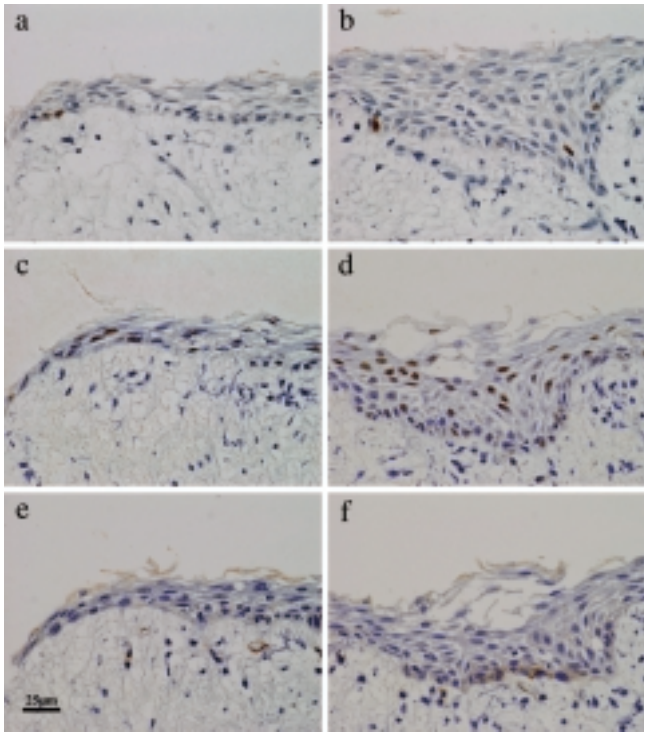

Fig. 2 Immunohistochemical expressions of BrdU (a, b), p63 (c, d) and $p75^{NGFR}$ (e, f) in the junctional epithelium. The left side is the apex of the junctional epithelium and the right side is the area neighboring the sulcular epithelium. The expression of $p75^{NGFR}$ is only seen in the basal layer neighboring the sulcular epithelium (e, f).

the sulcular epithelium, a small number of BrdU-positive nuclei were present in the suprabasal layer (Fig. 3a). In the oral gingival epithelium, BrdU-incorporating cells were mainly found at the side and base of the rete ridges in the basal layer (Fig. 4a: arrows; 4d: arrowheads). There was significant difference in the numbers of BrdU-incorporating cells in the three gingival epithelia ($P < 0.01$, Table 1).

Immunohistochemistry for p63 and p75^{NGFR}

p63-positive cells were present in the basal and superficial layers of the junctional epithelium (Figs. 1b. 2c, 2d). These cells were noticeable in the apical portions of the junctional epithelium, likely in BrdU-incorporating cells. In the sulcular and oral gingival epithelia, not only basal and suprabasal cells, but also a considerable number of cells in the lower spinous layers were positive for p63 (Figs. 3b, 4b, 4c). Particularly, in the apical portions of the rete ridges in the sulcular epithelium, almost all of the basal and suprabasal cells were immunostained with p63 antibody (Fig. 3b). Nuclei, which demonstrated an intense immunoreaction to p63, were present at the base and side of rete ridges in the oral gingival epithelium (Figs. 4b: arrows; 4c: arrowheads). As shown in Fig. 4, the immunohistochemistry for both BrdU and p63 revealed

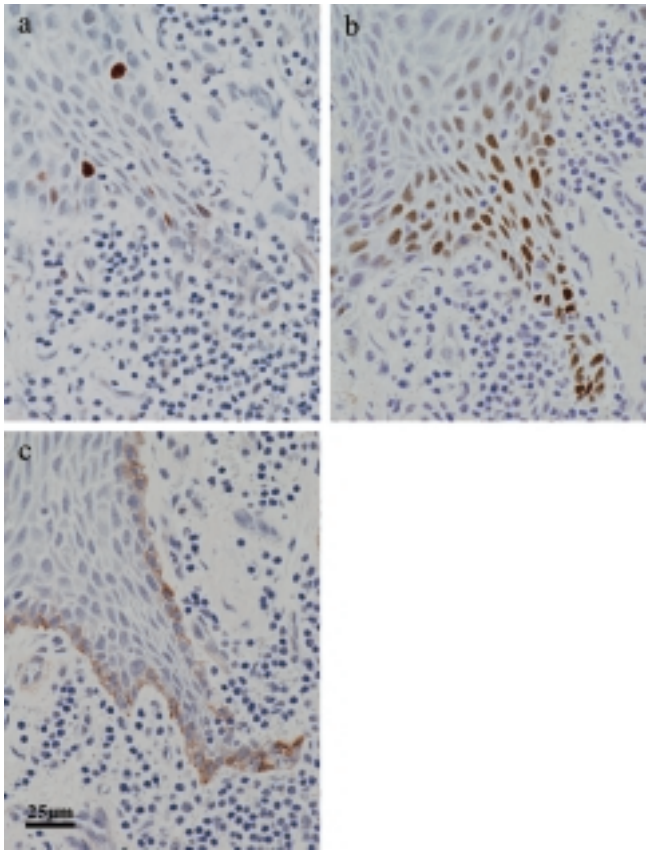

Fig. 3 Immunohistochemical expressions of BrdU (a), p63 (b) and p75^{NGFR} (c) in the sulcular epithelium magnified from respective parts shown in Fig. 1. BrdU-incorporating cells in the basal and suprabasal layers, and most basal cells are positive for both of p63 and p75^{NGFR}, although the intensity of the staining varied.

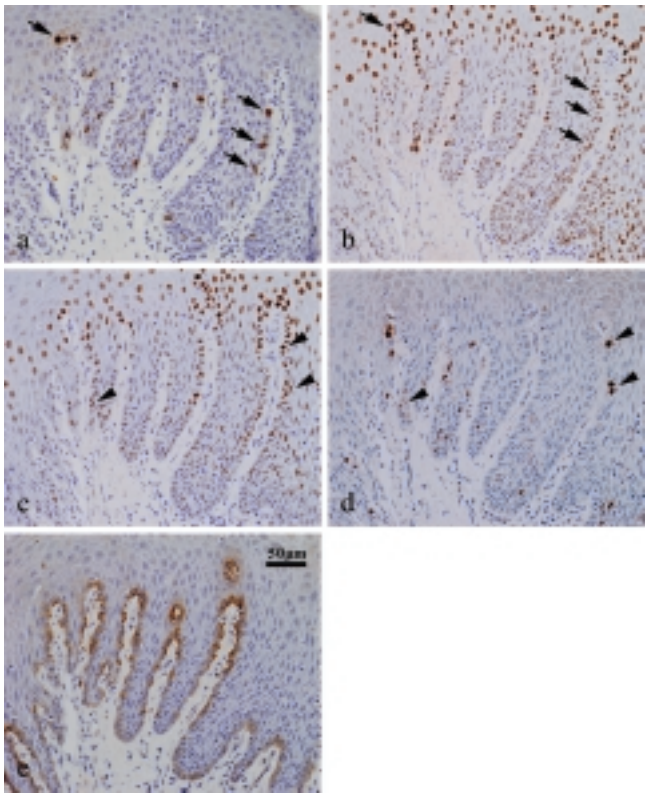

Fig. 4 The immunohistochemical expressions of BrdU (a, d), p63 (b, c) and p75^{NGFR} (e) in the oral gingival epithelium magnified. BrdU-incorporating cells are present in the basal layer, and p63 positive cells are observed in a broad area from the basal layer to the lower spinous layer, while p75^{NGFR} positive cells are restricted to the basal layer. These sections are continuous. Each distance is 16μm between section (a) and (b), 8μm between (b) and (c), 8μm between (c) and (d), and 24μm between (d) and (e).

Table 1 Proliferative activities of the gingival epithelium

	BrdU (n = 5)	
Oral gingival epithelium	10.96 ± 2.91	*
Sulcular epithelium	7.00 ± 3.14	**
Junctional epithelium	0.02 ± 0.03	**

Number of cells ×10 /mm, the mean ± SD
ANOVA: * $P < 0.05$, ** $P < 0.01$

that many p63 positive cells were present in the neighboring sections, where BrdU-incorporating cells were also observed.

$p75^{NGFR}$-positive cells were found in the basal layer, and were present in limited numbers near the sulcular epithelium in the junctional epithelium (Figs. 1c, 2e, 2f). The cell membrane of the basal cells was exclusively positive for $p75^{NGFR}$ in the sulcular and oral gingival epithelia (Figs. 3c, 4e). Stronger immunostaining for $p75^{NGFR}$ was observed from the side to base of the rete ridges than at the tip in oral gingival epithelium.

Discussion

Few investigations have analyzed the proliferative potential of cells in human gingival epithelium by using tracers such as [^{3}H]-thymidine (15,16) and by immunohistochemical detection of markers such as proliferating cell nuclear antigen (PCNA) (17). A few junctional epithelial cells attached to tooth were labeled by incubation for three hours in medium containing [^{3}H]-thymidine (15). However, the potential of incorporating [^{3}H]-thymidine in human junctional epithelial cells facing the connective tissue has not been clarified. The present study compared the frequencies of BrdU-incorporating cells in three portions of the gingival epithelium in human. The very low frequency of BrdU-labeled cells in the junctional epithelium indicated that it had no or very low proliferative activity. In contrast, the sulcular and oral gingival epithelia exhibited certain level of proliferative activity as shown in Table 1. From these results, it may be assumed that the junctional epithelium had generated from migrating progeny of proliferating cells in the neighboring sulcular epithelium.

p63, a homologue of the tumor-suppressor molecule of p53, is a transcription factor. Severe expressions of p63 in oral epithelial dysplasia (18) and squamous cell carcinoma with poorer survival rates (19) indicated that expression of p63 predicted high proliferative activity. Furthermore, stratified epithelia and their derivatives were absent in *p63*$^{-/-}$ mice (11,12) and that cells expressing p63 could be successfully transplanted (20). These observations led us to assume that p63 was closely associated with epidermal stem cells. However, it has not been determined yet whether p63-expressing basal cells are stem cells. Recently, it was reported that p63 was involved in maintenance of epidermal stem cells and stratification of the epidermis (21). The fact that cells rapidly adhering to collagen type IV successfully perform epidermal reconstruction (22) proved that epidermal stem cells were located in the basal layer. The p63-expression in a wide area covering the basal to spinous layers observed in the present study may explain the two functional roles of this protein-sustaining stem cells in the basal layer and stratification of the epidermis in the spinous layer.

The $p75^{NGFR}$ molecule is a low-affinity nerve growth factor receptor, which is a member of the TNF-α receptor superfamily, and its function is to mediate intercellular signaling in neural tissue, cell survival, and apoptosis (14). However, in human oral mucosal epithelium, immunohistochemical expression of $p75^{NGFR}$-expressing cells was found in the basal layer (23). In recent studies, $p75^{NGFR}$ was demonstrated to be a potential marker of esophageal keratinocyte stem cells (13) and oral keratinocyte stem cells (14). In the present study, cells with strong expression of $p75^{NGFR}$ were observed on the side near the base of the rete ridges in the oral gingival epithelium. Cells expressing abundant BrdU were also present in the same areas as $p75^{NGFR}$. These sites in the basal layer probably contained stem cells, which play an essential role in the supply of gingival epithelial cells. Ghazizadeh et al. (5) reported that stem cells were sparsely present along the basal compartment, and not in a certain limited area in the basal layer and each column generated by a single stem cell was called an "epidermal proliferative unit (EPU)". Furthermore, they observed that the widths of these columns vary considerably, with the narrowest one originating from the cells located at the base of the rete ridges. Therefore, stem cells were densely located at the base of the rete ridge. Our findings were in agreement with the above report.

Acknowledgments

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