Expression of p75<sub>NGFR</sub>, a Proliferative and Basal Cell Marker, in the Buccal Mucosa Epithelium during Re-epithelialization

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Received February 18, 2014; accepted May 23, 2014; published online July 31, 2014

We investigated the expression of p75<sub>NGFR</sub>, a proliferative and basal cell marker, in the mouse buccal mucosa epithelium during wound healing in order to elucidate the role of epithelial stem cells. Epithelial defects were generated in the epithelium of the buccal mucosa of 6-week-old mice using CO<sub>2</sub> laser irradiation. BrdU was immediately administered to mice following laser irradiation. They were then sacrificed after 1, 3, 7, and 14 days. Paraffin sections were prepared and the irradiated areas were analyzed using immunohistochemistry with anti-p75<sub>NGFR</sub>, BrdU, PCNA, and CK14 antibodies. During re-epithelialization, PCNA (–)/p75<sub>NGFR</sub>(+) cells extended to the wound, which then closed, whereas PCNA (+)/p75<sub>NGFR</sub>(+) cells were not observed at the edge of the wound. In addition, p75<sub>NGFR</sub>(–)/CK14 (+), which reflected the presence of post-mitotic differentiating cells, was observed in the supra-basal layers of the extended epithelium. BrdU (+)/p75<sub>NGFR</sub>(+), which reflected the presence of epithelial stem cells, was detected sparsely in buccal basal epithelial cells after healing, and disappeared after 7 days. These results suggest that p75<sub>NGFR</sub>(+) keratinocytes are localized in the basal layer, which contains oral epithelial stem cells, and retain the ability to proliferate in order to regenerate the buccal mucosal epithelium.

Key words: p75, buccal mucosa, wound healing, proliferation, stem cells

I. Introduction

The buccal mucosal epithelium is composed of a stratified squamous epithelium that is formed by multiple layers. Wound healing has been shown to occur faster in the buccal mucosa than that in skin and other epithelial tissues. Cell proliferation occurs principally in the basal layer of the epithelium in which keratinocytes undergo terminal differentiation as they migrate to the surface [9]. The basal layer of the stratified squamous epithelium consists of a heterogeneous population of proliferative and differentiating cells that are divided into three compartments and include transit-amplifying cells (TACs), post-mitotic differentiating cells (DCs), and epithelial stem cells (ESCs) [7, 11, 21, 25]. These cells have been shown to play a critical role in replenishing a regenerating epithelium [12, 23]. Previous studies also reported that human oral keratinocyte stem/progenitor cell phenotypes could be characterized by their expression of p75 as well as neurotrophin/p75 signaling [16, 17].

Potential candidates for epidermal stem cell markers, such as the low affinity nerve growth factor receptor p75 (p75<sub>NGFR</sub>), have been reported in the human cornea and epidermis [2, 8, 13, 14, 19, 26]. However, the localization and...
characteristics of these proteins during wound healing in the buccal mucosal epithelium have yet to be elucidated in detail.

Furthermore, the localization and role of epithelial stem cells need to be clarified during re-epithelialization. Therefore, we investigated the expression of p75NGFR in the buccal mucosal epithelium during wound healing in order to elucidate the role of epithelial stem cells.

II. Materials and Methods

All experiments were performed in accordance with the guidelines for experimental laboratory animals in the animal facility of Tokyo Dental College. Six-week-old ICR mice (n=40) were purchased from CLEA Japan, Inc. (Tokyo, Japan). They were divided into an irradiated group and non-irradiated group as a control. Five mice were used at each of the time points examined (1, 3, 7, and 14 days after laser irradiation).

Preparation of wounds in the buccal mucosa

The irradiation condition used was a continuous wave mode at a wavelength of 10,600 nm, 1W, using a 15-mm gauge contact tip (taper 1A), with an energetic density of 564.97 J/cm² using a CO₂ laser system (“Panalas C05Σ; Panasonic Shikoku Electronics Co., Ltd., Japan). The mouse oral mucosa between the upper and lower molars (5.0×5.5 mm) was irradiated for 10 sec using the top of the tip, which directly touched the irradiation area (Fig. 1a). One group of mice received laser irradiation at 6 weeks of age. After general anesthesia (intraperitoneal injection of sodium pentobarbital, 40 mg/kg body weight), saliva on the left side of the buccal mucosa was wiped with a cotton swab and this area was irradiated. BrdU (Sigma, St Louis, MO, USA) was injected intraperitoneally at a dose of 150 mg/kg of body weight immediately following laser irradiation. The same dose of BrdU was injected intraperitoneally into mice in the non-irradiated group.

Irradiated and control oral mucosa were excised along with portions of the surrounding tissue 1, 3, 7, and 14 days after irradiation. These samples were fixed in 10% neutral buffered formalin solution (Nacalai Tesque, Inc. Kyoto, Japan) overnight at 4°C, and were then demineralized with 10% ethylenediaminetetraacetic acid (EDTA, pH 7.0, Muto Pure Chemicals Co., Ltd. Tokyo, Japan) for 2 weeks at 4°C. These samples were embedded in paraffin following dehydration in ethanol, and were frontally sectioned at a thickness of 4 μm.

Two fields (Areas 1 and 2) were observed in the irradiated group, as shown in Figure 1b. The same positions on the opposite site were examined in the non-irradiated group.

Histological and immunohistochemical analyses

Paraffin sections from irradiated and control mice were deparaffinized with xylene and routinely stained with hematoxylin and eosin (H-E). Serial sections were also processed immunohistochemically. Briefly, sections were washed in phosphate-buffered saline (PBS) after paraffinization. Some of the serial sections for BrdU staining were incubated with 0.13 M sodium borohydride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 20 min to eliminate aldehyde autofluorescence, and were then washed for 10 min in PBS. The sections were then activated by boiling in 10 mM citrate buffer (pH 6.0) for 20 min. All sections were blocked with 10% normal donkey serum (Chemicon International Inc., Temecula, CA, USA) for 60 min at room temperature (RT). These sections were then incubated with BrdU (Sigma, St Louis, MO, USA) for 150 mg/kg of body weight immediately following laser irradiation. The same dose of BrdU was injected intraperitoneally into mice in the non-irradiated group.

Histological and immunohistochemical analyses

Paraffin sections from irradiated and control mice were deparaffinized with xylene and routinely stained with hematoxylin and eosin (H-E). Serial sections were also processed immunohistochemically. Briefly, sections were washed in phosphate-buffered saline (PBS) after paraffinization. Some of the serial sections for BrdU staining were incubated with 0.13 M sodium borohydride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 20 min to eliminate aldehyde autofluorescence, and were then washed for 10 min in PBS. The sections were then activated by boiling in 10 mM citrate buffer (pH 6.0) for 20 min. All sections were blocked with 10% normal donkey serum (Chemicon International Inc., Temecula, CA, USA) for 60 min at room temperature (RT). These sections were then incubated overnight at 4°C with BrdU (1:100 Abcam, Cambridge, UK), PCNA (1: 100 PC10, Novocastra Laboratories Ltd, London, UK), CK13 (1:100 Abcam), CK14 (1:100 COVANCE, San Diego, CA, USA), and p75NGFR (1:100 Abcam). These sections were incubated with AlexaFluor conjugated secondary antibodies (Invitrogen, San Diego, CA, USA) in the dark for 2 hr at RT, and then with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI, Dojindo Laboratories Inc., Osaka, Japan) for 5 min at RT. Double immunohistochemistry staining and an analysis for p75NGFR with PCNA and BrdU were also performed. Sections were observed under a fluorescent microscope (Axiophot 2, Carl Zeiss Oberkochen, Germany).

Assessment of immunohistochemically stained sections

Cells were considered positive if fluorescence staining was observed in the nucleus for PCNA and BrdU, or in the plasma membrane for p75NGFR (green color). All positively stained cells were scored as positive regardless of their intensity of staining. Cell counts were made at ×200 magnification under a conventional light microscope in 4 ran-
domly sampling fields from each mouse. The number of positive nuclei or cells was expressed as a percentage of the total number counted for the individual layers.

**Statistical analysis**

Group means for PCNA, BrdU, p75<sup>NGFR</sup>, PCNA/p75<sup>NGFR</sup>, and BrdU/p75<sup>NGFR</sup> were derived for each group. Data were analyzed using ANOVA and the Student-Newman-Keuls test.

### III. Results

**Histological observations**

The epithelium of the 6-week-old normal oral buccal mucosa was identified as the stratified squamous type, and well-developed dermal papillae were also observed (Fig. 2a). The epithelium in Area 1 as well as the normal oral epithelium was observed 1, 3, 7, and 14 days after irradiation (Fig. 2b). The epithelium with the lamina propria was lost through the entire thickness of the irradiated area 1 day after irradiation (Fig. 2b). The wound margin in Area 2 was observed 3 days after irradiation and epithelial elongation started at the edge of the wound margin toward the empty space (Fig. 2c). The wound margin in Area 2 was observed 3 days after irradiation and epithelial elongation started at the edge of the wound margin toward the empty space (Fig. 2c). Epithelial tissue grew from both sides of the wound margin at the irradiation area between 3 and 7 days after irradiation (Fig. 2c, d, m, n). The epithelium completely closed over the irradiated area after 14 days (Fig. 2e, o).

![Image of histological observations](image-url)
Localization of cytokeratins

CK 14 was only expressed in the basal layers of the epithelium in Area 1 and normal oral epithelium 1, 3, 7, and 14 days after irradiation (Fig. 3a–h). CK 13 was expressed in the supra-basal layers of the epithelium of Area 1 and normal oral epithelium (Fig. 3a–h). CK14 was only expressed in 1 layer of the basal epithelium in Area 2 one day after irradiation (Fig. 3i, m). However, the expression of CK14 was observed in 2–3 layers of the basal epithelium in Area 2 three, 7, and 14 days after irradiation (Fig. 3f–h, j–l, n–p). Very low CK 13 and CK 14 expression levels were observed in the leading edge of the outgrowing epithelium in Area 2 three and 7 days after irradiation (Fig. 3j, k, asterisks). The results obtained in the control area were similar to those in Area 1 (data not shown).

Localization of PCNA and BrdU

PCNA-positive (PCNA (+)) cells were observed in the basal layer in Area 1, Area 2, and the control one day after irradiation. The expression of PCNA 1 day after irradiation was similar to that in all areas 3, 7 and 14 days after irradiation (Fig. 4b–d, f–h), except for the leading edge of the outgrowing epithelium in the basal layer in Area 2 (Fig. 4b, c, asterisks). A significant difference was observed among all areas each day, except for 14 days (p<0.05), with a higher positive rate being noted in the wound area than in the control (Table 1).

BrdU-positive (BrdU (+)) cells were detected in the basal layer and supra-basal layer 1 day after irradiation in Areas 1 and 2 (Fig. 4i, m). Injected BrdU was incorporated into cells undergoing the cell cycle immediately after laser irradiation in all areas. BrdU (+) cells were detected in the basal and supra-basal layers in all areas 3 days after irradiation, including the leading edge of the outgrowing epithelium in Area 2 (Fig. 4j, n). Although a small number of BrdU (+) cells were observed in the control, they disappeared in the basal and supra-basal layers 7 and 14 days after irradiation in Areas 1 and 2, as well as the outgrowing epithelium in Area 2 (Fig. 4k, l, o, p). A significant difference was observed among all areas (p<0.01, Table 1), and a significantly higher positive rate was observed in the wound side than in the control 3 days after irradiation (Table 1). The results obtained in the control area were similar to those obtained 14 days after irradiation (data not shown).
Localization of p75NGFR

(p75NGFR (+) cells were observed in the basal layer in all areas each day after irradiation (Fig. 5a–h). p75NGFR (+) cells were also observed at the leading edge of the outgrowing epithelium 3 and 7 days after irradiation in Area 2 (Fig. 5b, c, f, g). A significant difference was observed in p75NGFR between all areas 1, 3, and 7 days after irradiation (p<0.01, Table 1). A significantly lower positive rate of

![Figure 4](image)

**Table 1**  Positive rate (%) of PCNA (+), BrdU (+) cells and p75NGFR (+) cells

|                | Area 1          | Area 2          | Control         |
|----------------|-----------------|-----------------|-----------------|
| PCNA (+)       |                 |                 |                 |
| 1 day          | 43.5 (±3.2)*1   | 37.8 (±8.0)*1   | 28.3 (±5.0)     |
| 3 days         | 40.5 (±9.1)*1   | 40.7 (±8.3)*1   | 28.8 (±3.7)     |
| 7 days         | 42.1 (±6.8)*1   | 34.1 (±6.6)*1   | 32.1 (±4.6)     |
| 14 days        | 38.5 (±9.1)     | 31.6 (±6.5)     | 31.6 (±4.0)     |
| BrdU (+)       |                 |                 |                 |
| 1 day          | 19.7 (±4.8)     | 17.0 (±7.5)     | 7.2 (±4.4)      |
| 3 days         | 21.5 (±7.9)*1   | 29.0 (±8.2)*1   | 12.3 (±4.2)     |
| 7 days         | N.D.            | 29.0 (±8.2)*1   | 12.3 (±4.2)     |
| 14 days        | N.D.            | N.D.            | 4.4 (±1.1)      |
| p75NGFR (+)    |                 |                 |                 |
| 1 day          | 23.5 (±2.4)*1   | 21.1 (±6.0)*1   | 31.7 (±2.8)     |
| 3 days         | 25.4 (±4.2)*1   | 27.3 (±6.0)*1   | 37.2 (±3.1)     |
| 7 days         | 35.8 (±4.6)*2   | 19.1 (±7.4)*1   | 29.3 (±3.7)     |
| 14 days        | 27.4 (±4.0)     | 29.1 (±7.7)     | 33.1 (±4.2)     |

Significant difference *1: Area 1 or Area 2 vs Control, *2: Area 1 vs Area 2
N.D.: not detected
p75NGFR was observed in the wound side, especially in Area 2, than in the control (Table 1). p75NGFR (+) cells were also expressed in the basal layer of the just contacted epithelial outgrowth area 14 days after irradiation in Area 2, into which both of the leading edges of epithelium cells extended (Fig. 5d, h). The results obtained in the control area were similar to those obtained 14 days after irradiation (data not shown).

**Localization of PCNA/p75NGFR and BrdU/p75NGFR**

PCNA/p75NGFR double positive cells (PCNA (+)/p75NGFR (+) cells) and PCNA-negative/p75NGFR-positive cells (PCNA (-)/p75NGFR (+) cells) were observed in the basal layer in all areas on all days after irradiation, (Fig. 6, arrow and arrowhead). However, PCNA (+)/p75NGFR (+) cells were not detected in the leading edge and the just contacted area at the leading edge of the outgrowing epithelium in Area 2 (Fig. 6, arrows).

BrdU/p75 double positive cells (BrdU (+)/p75NGFR (+) cells) were detected in the basal layer in all areas 1 day after irradiation, (Fig. 6i, m, arrowhead). A small number of BrdU (+)/p75NGFR (+) cells was detected in the basal layer 3 days after irradiation in Area 1 and in the outgrowing epithelium in Area 2 (Fig. 6j, n, arrowhead). Although BrdU (+)/p75NGFR (+) cells were detected in the control side 7 and 14 days after irradiation, they disappeared in the wound side (Fig. 6k, l, o, p).

The positive rate of PCNA (+)/p75NGFR (+) cells was significantly lower at 3, 7, and 14 days in Area 2 than in the control (p<0.01, Fig. 7). Significantly lower positive rates were observed in the wound side than in the control. Furthermore, a significant difference was observed in the positive rate of PCNA (-)/p75NGFR (+) cells at 14 days only between Area 2 and the control (p<0.01, Fig. 7). No significant differences were observed in the BrdU (+)/p75NGF (+) rate among Areas 1, 2, and the control.

**IV. Discussion**

The p75NGFR molecule is a low-affinity nerve growth factor receptor that is a member of the TNF-α receptor superfamily, and its function is to mediate intercellular signaling in neural tissue, cell survival, and apoptosis [16]. The localization of p75NGFR was previously detected in the basal layer of the human oral mucosal epithelium [4], and recent studies demonstrated that p75NGFR was a potential marker for keratinocyte stem cells in the esophagus [17] and oral cavity [16]. However, its function has not yet been elucidated. Wound healing is a model that can be used to understand the function of epithelial stem cells. Therefore, we focused on the expression of p75NGFR during wound healing in the buccal mucosa. In the present study, the expression of p75NGFR (+) cells was only observed in the basal layers of both the irradiated and control groups, while the number of p75NGFR (+) cells was reduced in the wound side from the early stage of wound healing. The number of p75NGFR cells recovered after the elongated epithelium completely closed over the irradiated area. A previous study reported that p75NGFR (+) cells exhibited the characteristics of epithelial stem cells [16]. Moreover, these cells were PCNA (-), which indicated non-proliferative cells. Our results suggest that regenerated and elongated epithelial cells may move from the wound margin to close the wound surface, but also that they are not proliferative and do not act as progenitor cells at the wound area.

In the present study, PCNA (+)/p75NGFR (+), which reflected the presence of stem cells, was observed at the basal cell layer during re-epithelialization. Self-renewing tissues such as the epidermis and intestinal epithelium contain both TACs and ESCs [1]. Therefore, two possibilities have been proposed concerning these cells. One is that the cells are TACs because the cells were proliferating, and the other is that they are true ESC [7, 11, 21, 25]. PCNA (+)
cells are proliferative cells that included epithelial stem cells during wound healing. However, TACs and ESCs are also proliferative during wound healing. Therefore, we used BrdU to discriminate TACs and ESCs. We hypothesized that ESCs would proliferate immediately after laser irradiation, and also that it would be possible to detect ESCs during re-epithelialization. We also hypothesized that ESCs may be scattered among unlabeled or weakly labeled cells as well as TACs in the tissue even after a period of several weeks [15, 27]. We did not detect p75 (+)/BrdU (+) cells 7 days after irradiation, although 0.9–1.8% of the epithelium was positive at the control side. These results imply

**Fig. 6.** Immunohistochemical observations of PCNA/p75^NGFR^ double staining and BrdU/p75^NGFR^ double staining in Area 2. (a–h) Immunohistochemistry for PCNA and p75^NGFR^ in Area 2 each day. (i–p) Immunohistochemistry for BrdU and p75^NGFR^ in Area 2 each day. (m–p) Enlarged images of (i–l), respectively. PCNA (+)/p75^NGFR^ (+) cells (arrowheads) and PCNA (−)/p75^NGFR^ (+) cells (arrows) were detected in the basal layer. BrdU (+)/p75^NGFR^ (+) cells (red and green: arrowhead) were detected in the basal layer of the outgrowing epithelium. Nuclei were stained with DAPI (blue). Bars=50 μm.
that the number of ESCs was low and needed more time to recover at the wound sites. However, further studies are needed to detect ESCs in wound sites.

CK14 is normally expressed in the basal layer, which is mainly composed of undifferentiated basal cells, of the stratified squamous epithelium containing the keratinized and non-keratinized epithelia of skin and oral mucosa [5, 18, 20, 22]. Furthermore, the expression pattern of CK14 has been shown to change in epithelial tissue during re-epithelialization [9, 24]. Epithelial stem cells have also been shown to express CK14 [3]. p75<sub>NGFR</sub> (+)/CK14 (+) cells may comprise epithelial stem cells. On the other hand, p75<sub>NGFR</sub> (–)/CK14 (+) cells were detected at the supra-basal cell layer in the present study. These cells may be DCs that progress to terminal differentiation.

In conclusion, the results of the present study demonstrated that p75<sub>NGFR</sub> (+) basal keratinocytes in the oral mucosa harbored the ability to migrate and regenerate the epithelium following an injury. p75<sub>NGFR</sub> (+)/PCNA (+) or BrdU (+) cells during the re-epithelialization process may contain ESCs in the oral basal keratinocytes. A schematic diagram of keratinocyte proliferation and migration in the wound epithelium was shown in Figure 8.
V. Acknowledgments

We would like to thank the staff at the Cornea Center (Ichikawa General Hospital Tokyo Dental College) for their technical support. This research was supported by Oral Health Science Center Grant HRC 7 from Tokyo Dental College, by a ‘High-Tech Research Center’ Project for Private Universities, and a matching fund subsidy from MEXT (The Ministry of Education, Culture, Sports, Science, and Technology) of Japan, 2006–2010.

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