

Re-epithelialization of the Buccal Mucosa after Alkaline Chemical Injury

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Alkaline conditions in the oral cavity may be caused by a variety of stimuli, including tobacco products, antacids, alkaline drinking water and bicarbonate toothpaste. However, the effects of an alkaline pH on the oral mucosa had not been elucidated. The purpose of this study was to investigate how basal keratinocytes are actively involved in re-epithelialization after alkaline chemical injury. We generated epithelial defects in the oral mucosa of mice by applying an alkaline chemical, and the localization of cytokeratin 13, cytokeratin 14, PCNA and p63 was investigated during the re-epithelialization process. PCNA- and p63-positive staining was seen in basal cells covering the wound surface at 1 day after the chemical injury. Cytokeratin 14-positive and PCNA-negative basal keratinocytes were localized in a few layers of the wound epithelium during epithelial outgrowth. Cytokeratin 14-positive and PCNA-positive basal keratinocytes, indicating proliferation, were localized over the entire layer of the epithelium at the wound margin. These results imply that basal keratinocytes at the wound margin migrate to the wound surface, provoke differentiation and keratinization during epithelial outgrowth and that epithelial cells are supplied from the wound margin to the epithelial outgrowth after alkaline chemical injury.

Key words: oral mucosa, wound healing, chemical injury, re-epithelialization

I. Introduction

The oral mucosal epithelium is affected by exposure to various extrinsic factors such as microorganisms and chemicals. Alkaline chemicals are generally known to induce injuries such as inflammation and erosions in the skin and mucosa. Transient alkalinity of the oral cavity may be brought about by a variety of factors, e.g. tobacco products, currently considered the most important causative factor of oral cancer. Indeed, the most common site of oral cancer in Taiwan is the buccal mucosa [5], while it is well known that a common site is the tongue in Japan. This is due to the

alkaline slaked lime in the betel quid in Taiwan [5]. The pharmacological effects of tobacco are related to alkalinity, due to the enhanced absorption of nicotine through the mucosa in the free-base form [26]. Alkaline substances are also used in various clinical settings such as bicarbonate toothpaste, calcium hydroxide materials for deep cavities and pulp, and sodium hydrochlorite for root canal treatment [27]. Therefore, it is possible that alkaline substances contact the oral mucosa accidentally, and it is important to understand reactions that result when that occurs. However, no experimental study on mucosal reactions to high alkalinity has been reported.

An alkaline shift in the extracellular environment may influence the growth properties of cells [6], and short-term alkaline stress may even cause proliferative activity [34]. However, cell proliferation and migration during the re-

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epithelialization process of the oral mucosa after alkaline chemical injury has not been well investigated. Prompted by an awareness of these alkaline conditions in the oral cavity, the objective of the present study was to investigate cell proliferation, migration and differentiation during the re-epithelialization process of the oral mucosa after application of NaOH, as a prototype of an alkaline chemical injury.

II. Materials and Methods

Materials

NaOH was purchased from Wako Pure Chemical Industries Ltd (Tokyo, Japan).

The mouse monoclonal antibody to PCNA was from Novocastra Laboratories Ltd. (London, UK), and rabbit polyclonal antibodies to p63 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), cytokeratin 13 (Abcam, Cambridge, UK) and cytokeratin 14 (Covance, San Diego, CA, USA) were used as primary antibodies.

The secondary antibodies, Alexa Flour 488 and 568 conjugated donkey anti-mouse IgG, were from Invitrogen (San Diego, CA, USA).

Experimental procedures

Post-natal (PN) 2-week old ICR mice (n=25) were purchased from CLEA Japan, Inc (Tokyo, Japan). All experiments were performed following the guidelines for the treatment of experimental animals at the Tokyo Dental College. The procedure was carried out according cornea injury model [16] with modification. After general anesthesia (intraperitoneal injection of sodium pentobarbital, 40 mg/kg body weight), the chemical injuries were caused by two 1 min exposures of the center of the buccal mucosa to a round piece of filter paper, 1.5 mm in diameter, soaked in 1 N NaOH, followed by rinsing with normal saline (10 ml). At the time when the oral mucosa was engorged with blood, we determined that the oral mucosa was injured. At intervals of 24, 48, 72, 120 hr and 1 week after chemical injuries, 10 samples were collected from 5 mice at each stage. As a control, untreated buccal mucosa was used.

Tissue preparation

Following sacrifice of the animals at different time points, the heads of the animals were perfused with physiological saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The heads were dissected and immersed in the same fixative for 24 hr at 4°C, and then were demineralized with 10% ethylenediamine tetraacetic acid (EDTA, pH 7.4) for 2 weeks at 4°C. The samples were embedded in paraffin and frontal-sectioned specimens were prepared (5- μ m). The sections were processed for routine hematoxylin and eosin (H-E) staining and immunohistochemical staining.

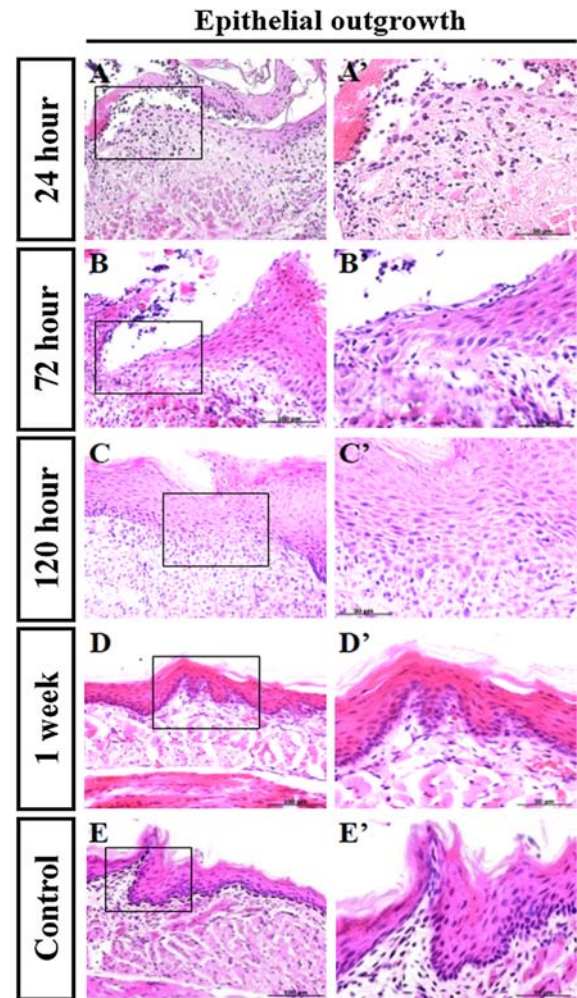


Fig. 1. Histological observations of chemically injured oral mucosa during epithelial outgrowth after 24 hr (A, A'), 72 hr (B, B'), 120 hr (C, C') and 1 week (D, D') after injury and in normal tissue (Control; E, E'). Higher magnifications of the boxed regions in A–E are shown as A'–E': respectively. The epithelium of the buccal mucosa was identified as the stratified squamous type. E, E': Epithelial elongation is observed from both sides of the wound margin toward the chemical injury. A, A', B, B': Re-epithelialization is observed in the degenerated muscle tissue. C, C': Regenerated epithelia from both sides of the wound margin are connected. D, D': The epithelium of the buccal mucosa has been completely regenerated and resembles the normal epithelium (E, E'). Bars=100 μ m (A–E), 150 μ m (A'–E').

Observation areas

We observed two areas, the wound margin and the outgrowth, during the wound healing process, as shown in Figure 1.

Immunohistochemistry

Serial sections were immunohistochemically processed. These sections were activated with 0.1 mM citrate buffer (pH 6.0) at 95°C for 20 min. Ten % normal donkey serum was applied to remove non-specific reactions. These specimens were incubated at 4°C with the primary anti-serum for 24 hr. After washing in PBS, the sections were

then incubated with the secondary antibodies in a dark box for 2 hr. Following counterstaining with 4',6-diamino-2-phenylidole dihydrochloride (DAPI; Invitrogen), all specimens were examined and photographed using a fluorescence microscope (Axio Vision, Carl Zeiss, Oberkochen, Germany).

Histological evaluation

Sections were stained with the indicated antibodies and were counterstained with DAPI to visualize all cells present in each section to enumerate the positive cells. Expression degrees of immunofluorescence were classified into the following stages. −; No staining, +; weak staining, ++; strong staining.

III. Results

Histological observations

At 24 hr after the chemical injury, inflammatory cells, including neutrophils and lymphocytes, appeared at the lamina propria, especially at the surface of the lamina propria (Fig. 1A, 1A'). Basal keratinocytes in the epithelial outgrowth were seen under debris and collagenous tissues (Fig. 1A, 1A'). The chemically injured epithelial cells became necrotic and no longer adhered to one another (Fig. 1A, 1A').

At 72 hr, epithelial tissues grew from both sides of the wound margin (Fig. 1B, 1B'), and the wound area was almost completely closed. Inflammatory cells were few in the wound epithelial tissue. However, the damaged muscle layers were extended, and many inflammatory cells were observed around these layers (Fig. 1B, 1B').

At 120 hr, the basal cells in the epithelial outgrowth had contacted each other, the damaged epithelium was completely closed and inflammatory cells were scarce in the wound epithelial tissues. The boundary of the regenerated epithelium and the underlying connective tissue was unclear. This regenerated epithelium was frequently found to harbor degenerated tissue and inflammatory cells (Fig. 1C, 1C').

At 1 week, the epithelium was completely closed, the basal layer of the regenerated epithelium was regular and the boundary of the regenerated epithelium and the underlying connective tissue was clear (Fig. 1D, 1D').

In the normal buccal oral mucosa of 2 week old mice, the epithelium was identified as the keratinized stratified squamous type, and well-developed papillae were observed. The tissue was attached to the underlying lamina propria. A thick layer of buccal muscle was observed beneath this mucosal layer (Fig. 1E, 1E').

Immunohistochemical observations

Localization of PCNA in the epithelium during the re-epithelialization process

At 24 hr after the chemical injury, PCNA-positive cells were observed at the epithelial outgrowth in the basal

layer (Fig. 2A, 2A'). At 72 hr, PCNA-positive cells were observed at the epithelial outgrowth in the basal layer as well as at 24 hr (Fig. 2B, 2B'). At 120 hr, PCNA-positive cells were observed near the contact area of the epithelial outgrowth where the leading edge of epithelial cells had migrated (Fig. 2C, 2C'). After 1 week, PCNA-positive cells were observed in the basal layer as well as in the normal epithelium (Fig. 2D, 2D').

Localization of p63 in the epithelium during the re-epithelialization process

p63-positive cells were localized in the basal layer of the normal oral mucosa (data not shown). At 24 hr after the chemical injury, p63-positive cells were observed at the leading edge of epithelial outgrowth in the basal layer (Fig. 2E, 2E'). At 72 hr, p63-positive cells were observed at the leading edge of the epithelial outgrowth (Fig. 2F, 2F'). At 120 hr, p63-positive cells were observed near the contact area where the leading edge of epithelial cells had migrated (Fig. 2G, 2G'). After 1 week, p63-positive cells were observed in the basal layer as well as in normal epithelium (Fig. 2H, 2H').

Expression of cytokeratins 13 and 14

In the normal oral mucosa, immunostaining for cytokeratin 14 was observed in the basal layer and other epithelial layers, including the suprabasal layers, did not show this immune-reactivity. Immunostaining for cytokeratin 13 was observed in the suprabasal layer of the normal oral mucosa (data not shown). At 24 hr after the chemical injury, cytokeratin 14 was expressed in all layers of the epithelial outgrowth, but cytokeratin 13 was not expressed in the epithelial outgrowth at all but was expressed in the debris of epithelium (Fig. 2I, 2I'). At 72 hr, cytokeratin 14 was expressed in the leading edge of the epithelial outgrowth (Fig. 2J, 2J'). Cytokeratin 13 was expressed in the suprabasal layer as it chased the edge of epithelial outgrowth that expressed cytokeratin 14 (Fig. 2J, 2J'). At 120 hr, cytokeratin 14 was expressed in the contact area where the leading edge of the epithelial epithelium had migrated (Fig. 2K, 2K') and cytokeratin 13 was expressed in the suprabasal layer as it chased cytokeratin 14 (Fig. 2K, 2K'). At 1 week, cytokeratin 14 was expressed in the basal layer and cytokeratin 13 was expressed in the suprabasal layer as well as in normal epithelium (Fig. 2L, 2L').

The expression of cytokeratins 13 and 14, PCNA and p63 during epithelial outgrowth are summarized in Table 1.

IV. Discussion

The wound healing process of the skin has been investigated morphologically [10, 19, 23]. Earlier studies showed that regenerative epithelial cells migrate from the wound margin [10, 23]. The proliferative activity of the regenerative epithelium has also been investigated by radio-autographic labeling with tritiated thymidine, and labeled cells were frequently seen at the wound margin at 1 day after injury [1–3, 8]. Histologically wound healing after

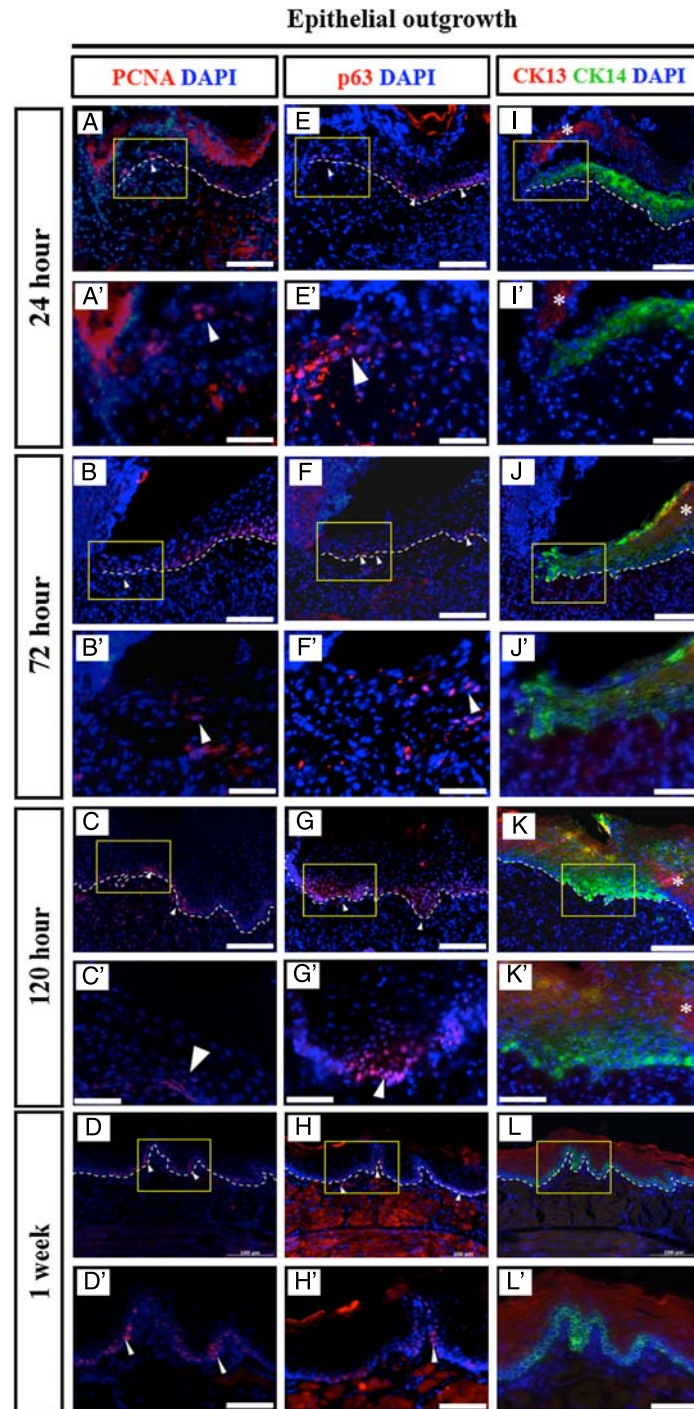


Fig. 2. Immunohistochemical observations of PCNA, p63 and cytokeratins 13 and 14 in normal tissue and in epithelial outgrowth of the chemically injured oral mucosa. **A, A', B, B'**: After 24 hr and 72 hr, PCNA-positive cells are observed at the leading edge of the epithelial outgrowth in the basal layer (*white arrowhead*). **C, C'**: After 120 hr, PCNA-positive cells are observed near the contact area of the epithelial outgrowth where the leading edges of epithelial cells migrate towards each other (*white arrowheads*). **D, D'**: After 1 week, PCNA-positive cells are observed in the basal layer as well as in normal epithelium. **E, E', F, F'**: After 24 hr and 72 hr, p63-positive cells are observed at the leading edge of epithelial outgrowth in the basal layer as well as PCNA-positive cells (*white arrowhead*). **G, G'**: After 120 hr, p63-positive cells are observed in the center of the contact area where both leading edges of epithelial cells have migrated toward each other (*white arrowheads*). **H, H'**: After 1 week, p63-positive cells are observed in the basal layer as well as in normal epithelium. **I, I'**: After 24 hr, cytokeratin 14 is expressed in all layers of the epithelial outgrowth, but cytokeratin 13 is not expressed in the epithelial outgrowth at all. Cytokeratin 13 is expressed in the debris of the epithelium (*asterisks*). **J, J'**: After 72 hr, cytokeratin 14 is expressed in the leading edge of the epithelial outgrowth, and cytokeratin 13 is expressed in the suprabasal layer as it chases cytokeratin 14 (*asterisks*). **K, K'**: After 120 hr, cytokeratin 14 is expressed in the contact area where both leading edges of the epithelial epithelium migrate towards each other and cytokeratin 13 is expressed in the suprabasal layer as it chases cytokeratin 14 (*asterisks*). **L, L'**: After 1 week, cytokeratin 14 is expressed in the basal layer and cytokeratin 13 is expressed in the suprabasal layer as well as in normal epithelium. *White dotted line* in **A–O, A'–L'** demarks the basement membrane. Bars=100 μ m (**A–O**), 50 μ m (**A'–L'**).

Table 1. Expression of cytokeratin13/14, PCNA, Ki67 and p63 on epithelial growth during wound healing

	24 hr	72 hr	120 hr	1 week
CK13	–	–	+	++
CK14	++	++	++	+
PCNA	+	+	+	+
p63	+	++	++	+

The expression degrees of CK13/14, PCNA and p63 were classified. –: Negative, +: Weak positive, ++: Strong positive.

alkaline injury was similar to that after incision. However, it had not been fully investigated whether the proliferation of basal keratinocytes occurs during wound healing in the oral mucosa, and therefore, we used immunohistochemistry with PCNA and p63 as markers for proliferative cells and basal cells, respectively. Our results show that PCNA/p63 double-positive cells are not seen at the outgrowth area at 1 day after the alkaline chemical injury, while they are localized in the basal cell layer in the normal epithelium, as had been reported earlier [33]. p63-positive basal cells act as progenitors of suprabasal cells, which undergo differentiation in regenerative epithelia [11, 29]. Our results suggest that regenerative epithelial cells move from the wound

margin to close the wound surface at 24 hr after injury, but they are not proliferative and do not act as progenitor cells at the wound area. Furthermore, p63 (+) cells were seen in multi-layers at 1 week after injury. Previous reports have described p63 is localized to hyperproliferative keratinocytes, including basal and suprabasal keratinocytes, in the stratified epithelium [20]. Moreover, p63 may be a suitable marker for undifferentiated cells of the epithelium [22]. The finding suggests that regenerative cells in the area may be hyperproliferative and undifferentiated cells.

Cytokeratin, one of the intermediate filaments, is a crucial marker to evaluate epithelial differentiation [18] and is involved in wound healing [31]. Cytokeratin expression

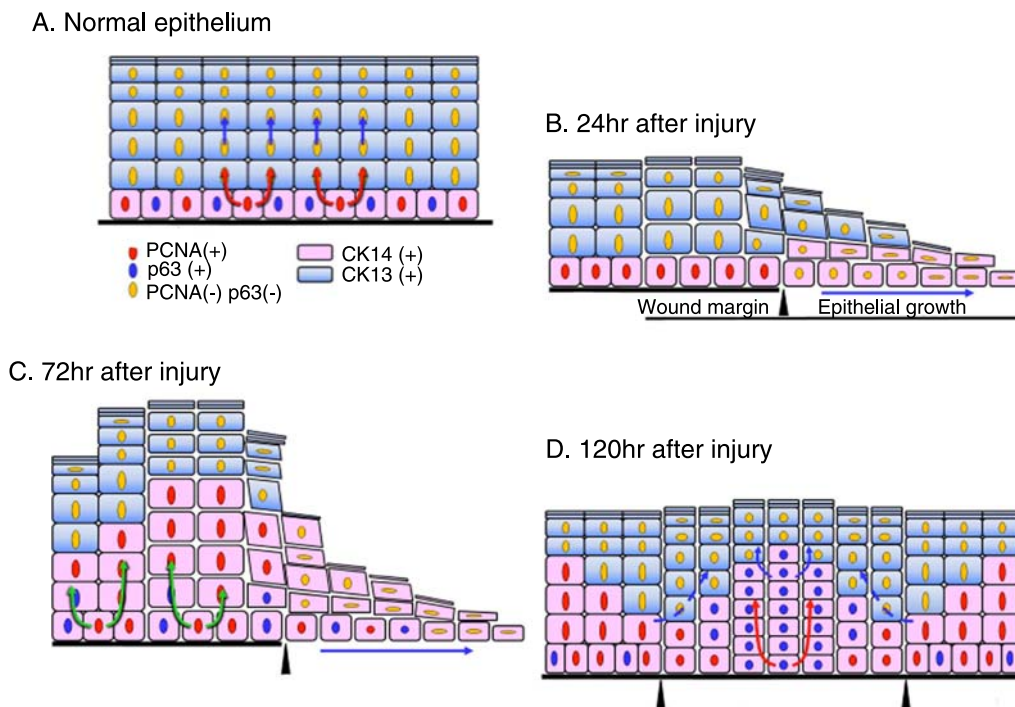


Fig. 3. Schematic diagram of normal epithelial characteristics and keratinocyte proliferation and migration in normal (A) and wound epithelium (B, C, D). **A:** Immunohistochemical localizations of PCNA, p63 and cytokeratins 13 and 14, in the normal tissue are summarized. Red nuclei represent PCNA-positive cells. Blue nuclei represent p63-positive cells and yellow nuclei represent PCNA and p63-negative cells. The pink color box shows cells cytokeratin 14-positive cells in the basal keratinocytes and the sky blue color box shows cytokeratin 13-positive cells in the suprabasal basal keratinocytes. **B:** At 24 hr after the wound injury, the basal keratinocytes are activated to migrate and lead the edge of the wound epithelium. However, PCNA- and p63-positive cells are only seen in the basal layer of the wound margin and in normal epithelium, and are not observed in the epithelial outgrowth of the wound epithelium. **C:** At 72 hr after the wound injury, as basal keratinocytes surrounding the wound margin evidence a positive reaction for PCNA and express cytokeratin 14, these cells are proliferating and contribute to the source of cells available for wound closure. **D:** At 120 hr after the wound injury, the majority of cells in the regenerated epithelium are positive for p63, thus indicating that the majority of the regenerated epithelium is formed by basal keratinocytes that have migrated from the surrounding epithelium. A small number of keratinocytes in the regenerated epithelium are negative for p63, which suggests that these cells may have originated from migrating basal keratinocytes or from original suprabasal keratinocytes.

has been studied in dermal keratinocytes during wound healing using immunohistochemistry [8, 13, 14, 17, 21, 30]. Cytokeratin 13 is distributed in the suprabasal layers of stratified epithelia and cytokeratin 14 is mainly expressed in undifferentiated basal cells of stratified squamous epithelium [9, 25]. An earlier report showed that expression of cytokeratin 14 is observed in the regenerative cells attached to the fibrin net of the wound area caused by gingivectomy [28]. Our results demonstrate that basal and suprabasal keratinocytes show intense expression of cytokeratin 14, resembling non-keratinized cells, at 24 hr after the injury. Also, the same expression patterns of cytokeratins 13 and 14 as that seen in healthy epithelium were seen at 7 days after the injury. These findings suggest that differentiation and keratinization are stimulated at the epithelial outgrowth during wound healing after the injury.

Our results show that the wound becomes crater-shaped grossly and microscopically at 72 hr, because the wound margin rose up, and subsequently, the surface of the wound became flat. Our immunohistochemical observations also show that PNCA- and cytokeratin 14-positive cells are frequently seen at the wound margin. These findings imply that wound healing is provoked not only by the proliferation and differentiation of basal cells but also by supplying cells from the wound margin to the epithelial outgrowth. Two models, a “rolling” model and a “sliding” model [12, 15] have been proposed to occur during wound healing of the skin. In the “rolling” model, the primary keratinocytes entering the wound area originate from the suprabasal layers. Suprabasal keratinocytes, when exposed to the wound environment, are believed to alter their shape and to tumble over basal keratinocytes attached to the basement membrane. The “sliding” model emphasizes basal keratinocytes at the wound margins. These basal keratinocytes are considered to migrate laterally over the provisional wound matrix and to pull at the remaining epithelium. Suprabasal keratinocytes perform an ancillary function in this model [7, 24, 32]. Additionally, recent studies have reported that both basal and suprabasal keratinocytes may constitute the principal source of cells available for wound closure [21, 30]. Therefore, the re-epithelialization process of the wound epithelium remains controversial. Our immunohistochemical results imply that both “sliding” and “rolling” occur during the wound healing. However, further detailed investigation, such as a BrdU chase study carried out by Bickenbach [4], is necessary to confirm the change of characteristics during wound healing.

In conclusion, our results imply that basal keratinocytes at the wound margin migrate to the wound surface, provoke differentiation and keratinization during epithelial outgrowth and that epithelial cells are supplied from the wound margin to the epithelial outgrowth after alkaline chemical injury, as shown schematically in Figure 3.

V. Acknowledgments

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