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The Temporal and Spatial Expression of the Cytokeratin in Keratinocytes during Cutaneous Wound Healing on the Amphibian (*Bombina orientalis*)

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양서류 피부 상처회복과정 중 각질화세포 cytokeratin의 분포

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ABSTRACT

The system of wound healing is very complex biological processing that includes inflammatory, reepithelialization, and matrix construction. For identification of the transitional pathway of the keratinocytes, we have employed immunohistochemical analysis using cytokeratin antibody after wounding. Epithelium in skin of the frog (*Bombina orientalis*) was examined with transmission electron microscopy. Cytokeratin was expressed in normal basal and gland cavity cells. At 3-hour basal layer cells were strong positive, however cells of the upper layer were negative reaction. Day1 and 2 after post-wounding, regenerating epithelial cell layer was positive reaction, especially basal layer cells were strong positive. At day 10 after wounding, the degree of positive reaction to basal cells of regenerating epithelial tissue was equal to day 7 wound tissue. At day of 19th, basal and spinous layer cells were strong positive reaction. Regenerating epithelial cells were positive but some basal cells were strong positive at day 27. From this result, we identified that the migration of the keratinocytes in amphibian skin wounds is initiated from basal layer cells and the keratinocytes migrate into basal and middle of the wound area.

Keywords : Amphibian, *Bombina orientalis*, Cutaneous, Cytokeratin, Keratinocyte, Wound healing

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INTRODUCTION

During the healing of the cutaneous wounds, epidermal repair is manifested by the progressive extension of a stratified sheet of epidermal cells from the peripheral epidermis across wound. Changes in the presence and distribution of the maturation markers in human epidermis following injury have been studied with the goal of understanding the nature of keratinocytes responses to environmental stress. From these reasons, we have utilized polyclonal antibody (Pan-cytokeratin) in attempts to elucidate the migration of the wound epithelium in regeneration.

Research on reepithelialization of the wounds has been centered mainly on vertebrates, especially mammals, and has been revised by Stenn (1986). Studies of the wound healing in Oligochaeta have been reviewed by Cooper & Roch (1984). With regard to leeches, published work on wound healing is limited to some brief descriptions made by LeGore & Sparks (1971, 1973); Cornec & Coulomb-Gary's paper (1982) on regeneration of amputations; and a study made by the authors on the process of healing in Hirudo (Huguet & Molinas, 1992).

Changes in the presence and distribution of maturation markers in epidermis following injury have been studied with the goal of understanding the nature of keratinocyte responses to environmental stress (Fuchs & Green, 1980). The present work is a study on the ultrastructure of the skin and immunohistochemial level of the migration of the keratinocytes in the wound healing process of *Bombina orientalis*. The morphology and remodeling of the epithelial cells has been described in precious works by authors (Jeong & Moon, 1998, 1999).

This work is to describe changes in cell morphology which occur during cell migration and to reveal the pattern of the involved locomotion. The migratory behavior of *Bombina* epithelial cells during the wound healing is compared with the models of epithelialization examined by others, we also immunohistochemically monitored the expression of the cytokeratin as known epidermal maturation marker in skin-removed wounds up to 1-month.

MATERIALS AND METHODS

1. Animals

Adult frogs, *B. orientalis*, were collected in Mt. Wangbang at Gyeong-do Korea, maintained in aged tap water at room temperature, and fed beetle twice weekly. Pieces of the skin 2.0 ~ 4.0 mm^2 were dissected from the dorsal surface of frog with a razor blade. Frogs were then returned to water and allowed to regenerate to the desired stage. Regenerates were collected at varying intervals post-injury: 0 hr to 24 and 48 hr; day 3 to day 14; 1 month, respectively. Ten frogs in each group were used. About 2/5 of the sample was used for transmission electron microscopy, 3/5 for immunohistochemistry.

2. Histochemical procedures

For immunohistochemical demonstration of the keratin protein, the samples were fixed 4% paraformaldehyde, pH 7.2 at 4°C for 4 hr. Then the tissues were washed with PBS and dehydrated by an ethanol grade series. To eliminate nonspecific reaction, they were dehydrated with absolute methanol and infiltrated and embedded with Paraplast after clearing of Histo-Clear (national diagnostics, USA) at 56 ~ 57°C. The 6 to 7 μm sections were mounted on Ploy-L-Lysin (Sigma) coated slides. To remove the nonspecific binding of antibody (unmasking), sodium citrate (0.1 M, pH 6.0) was applied to slide for 5 min and to inhibit activity of peroxidase 3% hydrogen peroxide was applied.

Anti-mouse-pan cytokeratin (monoclonal, Sigma Immunochemical Co., USA) as primary antibody used at the ratio of 1 : 400 and incubated 1 hr at humidity chamber. After washing with PBS, biotinylated goat anti mouse IgG (DAKO Co., USA) was applied using avidin

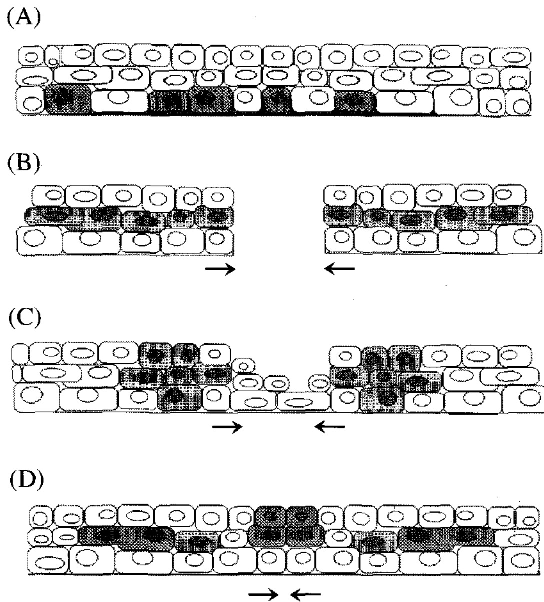

Fig. 1. Local changes of the keratinocytes in the epithelium during the wound healing. Meshed cells indicate strongly immunoreactive keratinocytes. A: normal; B: 6 hours; C: 12 hours; D: 1 to 3 days.

-biothin complex system (Guesdon et al., 1979) for 30 min. Strepta-avidin peroxidase (DAKO, Co., USA) was incubated for 10 min and then applied DAB (diaminobenzidine tetrahydrochloride, Sigma, Immunochemical Co., USA) (0.03% hydrogen peroxide, 0.05 M Tris-HCl) for 5 min. After reaction the slides were mounted with Permount (Fisher) and observed.

For transmission electron microscopy, specimens were subjected to a double fixation. The first, they were fixed with Karnowsky fixative for 2 hr at 4°C, washed in phosphate buffer, pH 7.4 and then post-fixed in 1% in osmium tetroxide in the same buffer for 1 hr. Then the specimens were dehydrated through a graded ethanol series, exchanged through propylene oxide, and embedded in a mixture of Epon and Araldite (Polysciences). Semi thin (2 μm) sections were stained with toluidine blue and viewed in the photomicroscope. Selected areas of the embedded tissue were thin-sectioned on Reichert Ultramicrotome. Thin sections were stained with uranyl acetate and lead citrate and viewed in a JEOL CXII electron microscope at operated at 80 kV.

RESULTS

In normal dorsal skin, the basal layer was consisted of the columnar or cuboidal cells, each cell of which has short, thin, cytoplasmic processes on its surface. The cell membrane with relation to the basal lamina exhibited numerous hemidesmosome (Fig. 2a). The spinous layer was composed of the polyhedral cells, slightly separated from each other (Fig. 2b). The basal cells contained bundles of the fine filaments randomly distributed throughout the cytoplasm. Desmosomes were frequently found at the lateral and upper surfaces of the cells (Fig. 2c). The cytoplasm of granular layer cell contained granules of keratohyalin which appeared irregularly shaped masses of the electron dense material in association with bundle of filaments (Fig. 2d).

Control tissue was stained with the omitting first antibody, showing no staining except nucleus for counter staining (Fig. 3a). In normal tissue, basal layer of the epidermis was strong intensity but not suprabasal layer (Fig. 3b). In addition, cells consisting of gland were strongly reacted with cytokeratin antibody (Fig. 3c). One hour after wounds, the staining showed weakly intensity including basement membrane and detached basal layer cells (Fig. 3d). The epithelial cell near by wound margin, at 3 hour, the shape of the cells involving the migration to the wound was changed to oval. There was no reaction on the suprabasal layer cells but not basal layer cells (Fig. 3e). After 6 hour, the suprabasal layer cells were stained with cytokeratin antibody though the reaction was weak, intriguingly, the oval shape cells came from basal layer cells were strong reaction around their nucleus (Fig. 3f).

The initiation of the epithelial cells to the wound area was observed at 12 hours after wounding and the cells upper suprabasal layer were detached from cells lower

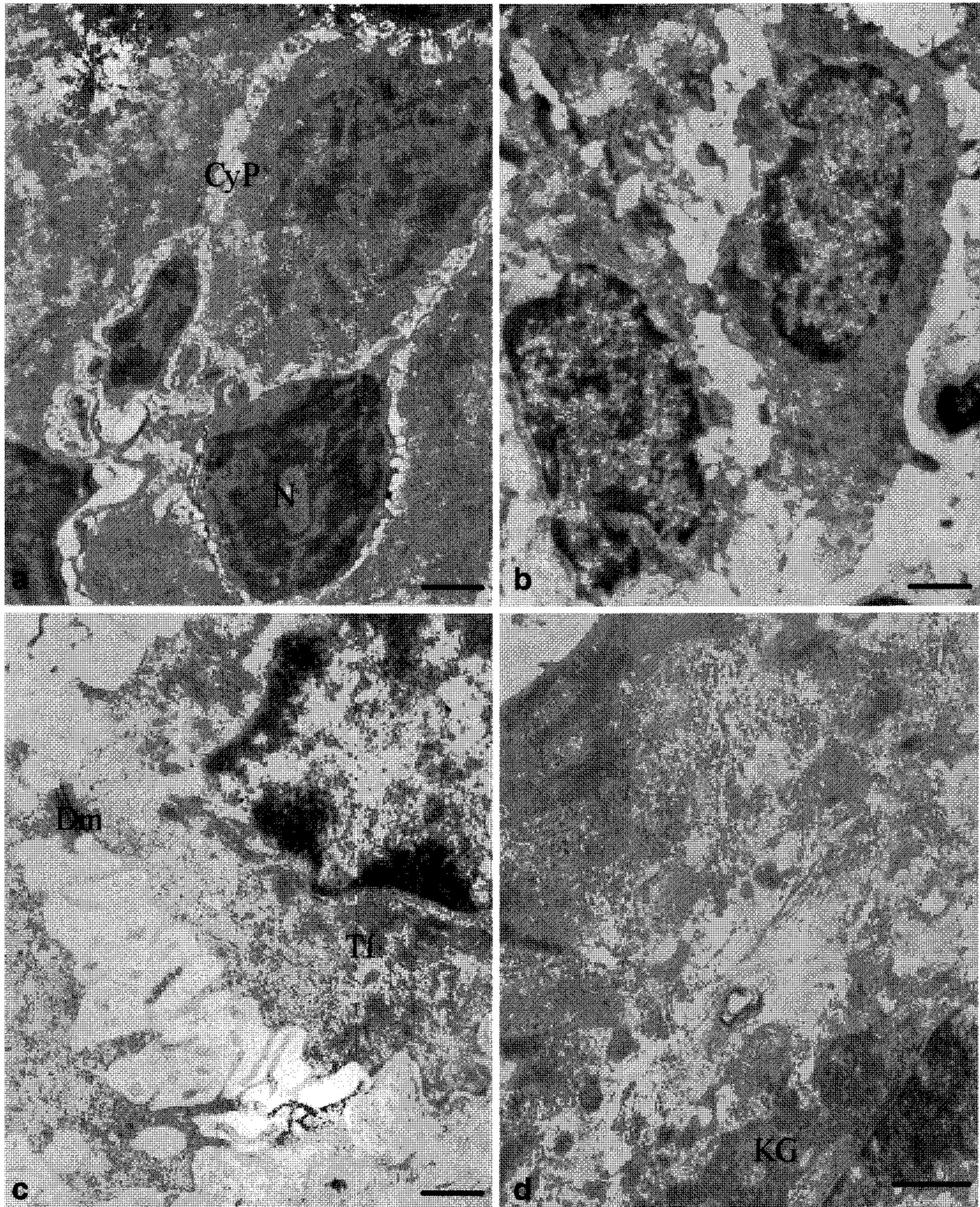

Fig. 2. Normal epidermal layer. a: The basal layer consists of columnar or cuboidal cells which have cytoplasmic processes (CyP) on its surface. N, nucleus. Scale bar is 2 μm. b: Numerous polyhedral cells in spinous layer are slightly separated. Scale bar is 2 μm. c: Bundles of the fine filaments randomly distributed throughout the cytoplasm are observed in the basal cells. Dm, desmosomes; Tf, tonofilament. Scale bar is 1 μm. d: Granular layer cells contains granules of keratohyalin, which appear irregularly shaped masses of the electron dense material. KG, keratohyalin granule. Scale bar is 1 μm.

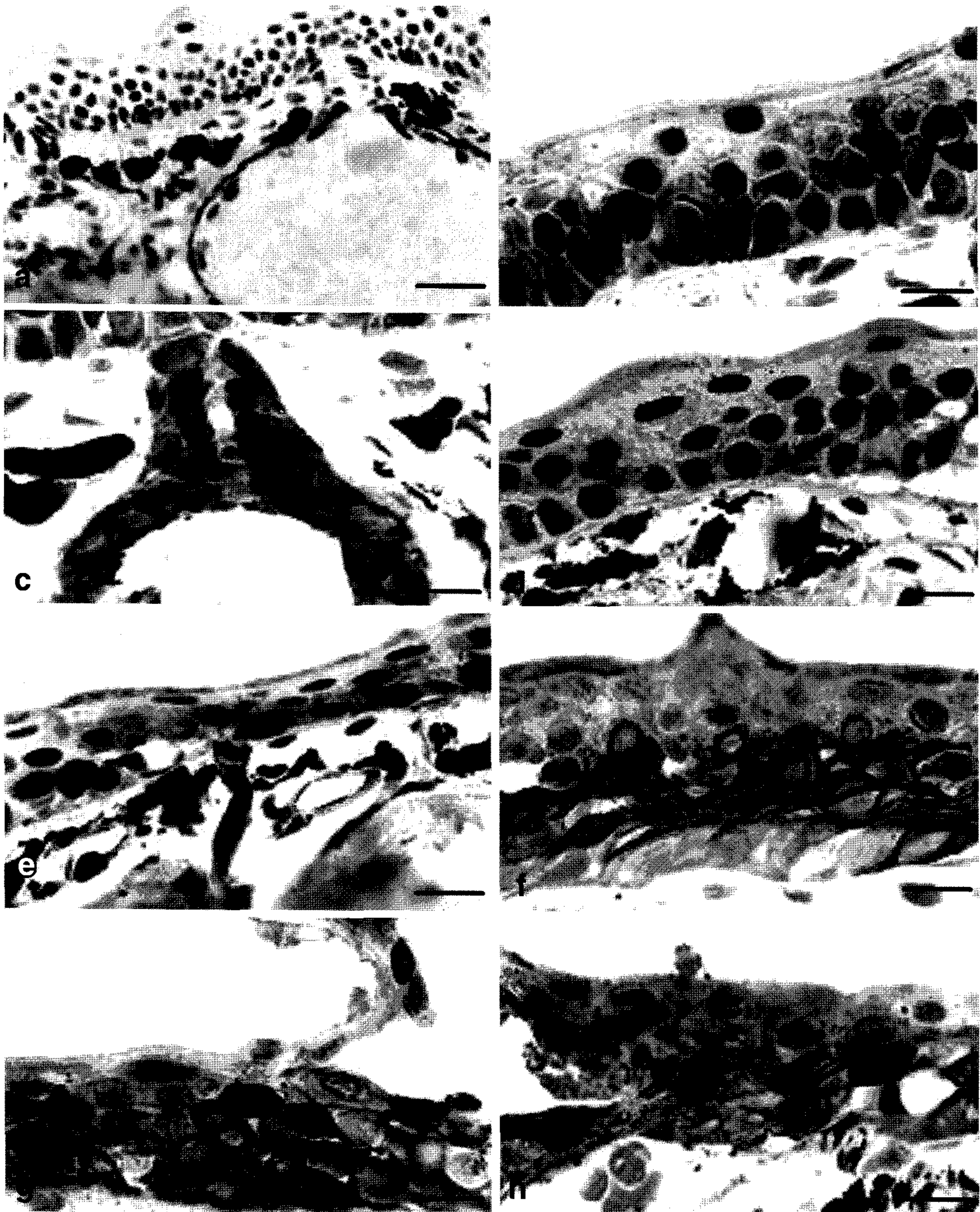

Fig. 3. Expression of cytokeratin on the early healing responses. a: In control staining, normal skin reacts as negative reaction. Scale bar indicate 50 μm. b, c: Basal cells and gland cavity cells appear as a strong positive with cytokeratin antibody. d: After 1 hour, basal layer cells are deep straw color. e: At 3 hours basal layer cells are strong positive, but upper layer cells are negative reaction. f: Cytoplasm near the nucleus in basal cells is stained deep straw color at 6 hours after wounding. g, h: At 12 hours, epithelial cells as shown strong positive spread over the wound surface. These cells participating in wound closure are originated from the adjacent epidermis. Scale bars from b to h indicate 10 μm.

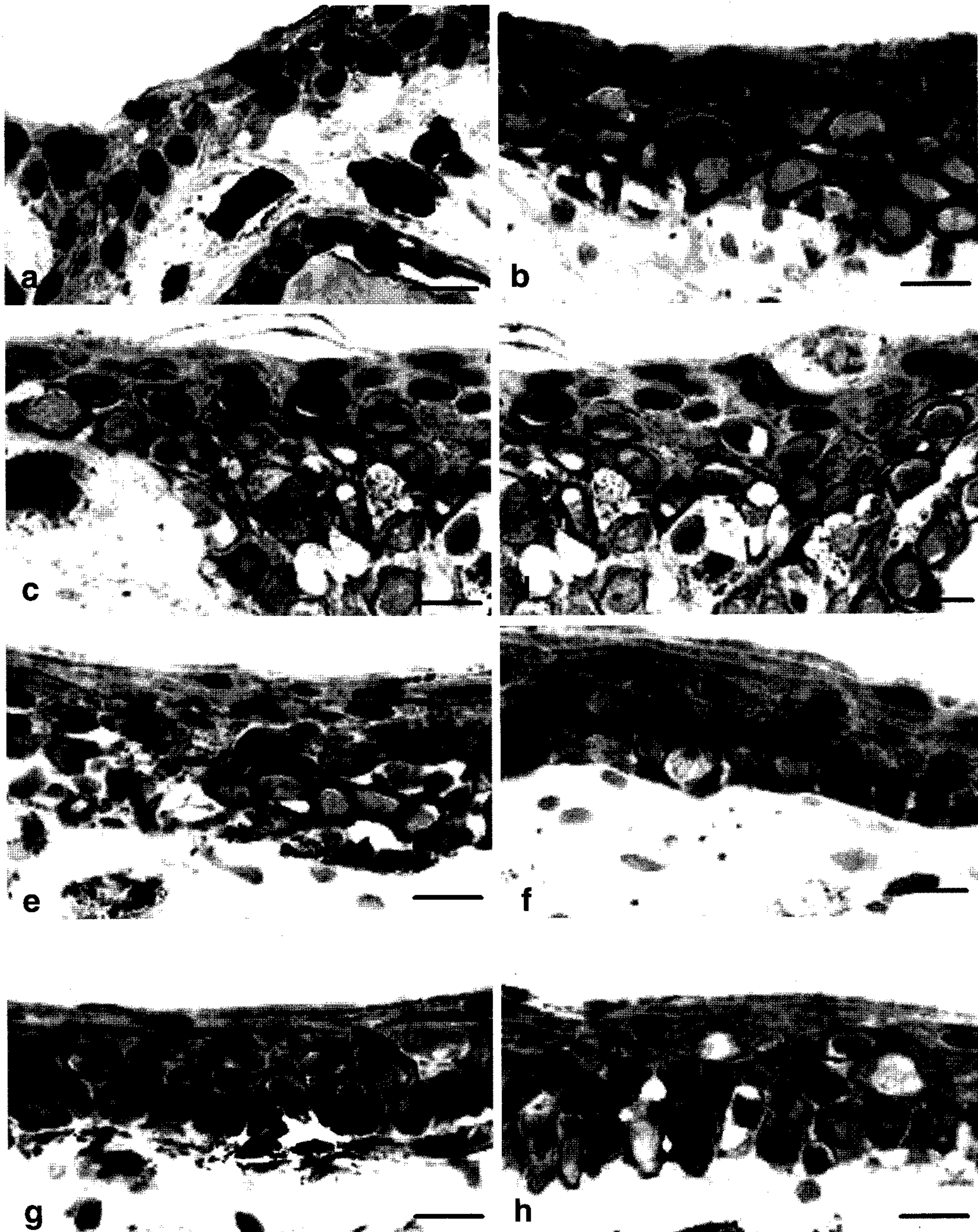

Fig. 4. Immuno-reaction of the cytokeratin antibody during the wound healing responses. a, b: At day 1 and 2, regenerating epithelial cells show positive reaction. Especially basal cells are strong positive. c, d: Regenerating epithelial cells are medium reaction at day 4. The cells of the middle region are strong positive. e, f: At day 10, the degree of positive reaction to basal cells of regenerating epithelium is equal to day 7 tissue. g: The horny and clear layer are strong straw color at 16 days. At day of 19th, basal and spinous layer cells are strong reaction. h: At 27 days post wounding, regenerating epithelial cells are positive but some basal cells show strong positive reaction. All scale bars indicate 10 μm.

layer. The basal layer at the wounds edge and near the wounds showed strong positive reaction compared with the other cells (Fig. 3g, h). After 1 day the migration of the epithelial cells was still observed in wound area and wound closure by regenerated epithelial cells was observed at day 2. All of the cells engaged in re-epithlialization were strong intensity; especially the cells of basal region were more, suggesting the cells involved in migration in wound are mainly keratinocytes from origin of basal layer (Fig. 4a, b).

In regenerated cells (covered wound area), medium intensity was observed overall except at the middle of this having a strong intensity at day 4 after wound (Fig. 4c, d). At the margin of the wound, basal layer was strong intensity during day 7 to 10 (Fig. 4e, f). In observation for day 16, upper region of the basal layer was strongly, but tissue of the day 19 showed strong intensity on the basal- and spinous layer (Fig. 4g). At the last day of the observation (day 27), all of the regenerated cells showed slightly positive reaction except several cells of the basal layer (Fig. 4h).

In summary for the expression of the cytokeratin, basal layer cells of the normal tissue were strong positive reaction, which was similar to the wound tissue immediately after injury. According to the temporal and spatial expression of the cytokeratin, it was strong expressed in basal layer cell at 6 hr and in middle layer cells near the wounds at 12 hr after injury. Between 24 hr and day 4, regenerated cells toward to below of the wound area and the cells that were located in the middle of the wound area after wound closure were strongly expressed (Fig. 1). From this result, we showed that the migration of the keratinocytes in amphibian skin wounds is initiated from basal layer cells, according to the time goes by, the keratinocytes migrate into basal and middle of the wound area.

DISCUSSION

Cytokeratin is a family of the proteins with molecular weights ranging from 40 to 70 kDa which are responsible for formation of tonofilaments within the epidermal keratinocyte (Moll et al., 1982). Cytokeratin has been demonstrated to be sensitive marker of keratinocyte differentiation and their distribution pattern in the human epidermis has been observed to be modified in pathological and experimental conditions (Schlegel et al., 1980; Woodcock-Mitchell et al., 1982). In the present study, using this method we have detected the spatial and temporal expression of cytokeratin during healing process. Thus from this result, we have also chased the pathway of keartinocytes (Fig. 1).

Several *in vitro* studies have shown that cultured epidermal keratinocytes resemble epidermis *in vivo* in the ability to regenerate the suprabasal layers after injuy, and the overall *in vitro* epidermal response to stripping is very similar to the *in vivo* responses (Jensen & Bolund, 1988; Read & Watt, 1988).The morphology of the regenerated epidermis has already been described in cultured human skin. According to this, after 1 week a two to four layered epithelium with flattened cells containing spindle-shaped nuclei in the upper layers (Moll et al., 1998). Similarly, in our experiment *in vivo*, regenerated epithelium was composed of the flatten cells and basal cells which showed strong positive. Taken together, these results showed that basal or suprabasal layer cells can lead to reepithelialization during the wound healing.

In our results, the same expression of the cytokeratin in migrating epithelium as that in basal keratinocytes are compatible with the fact that only basal keratinocytes divide and proliferate in normal epidermis. In previous studies, however, it was reported that not basal keratinocytes but suprabasal keratinocytes appeared to move by rolling or sliding over the basal layer below (Sciubba, 1977; Gibbins, 1978). Based on the immunostaining, some investigator concluded that basal cells do not migrate laterally, in contrast to suprabasal keratinocytes (Ortonne et al., 1981). However, a similar staining pattern in concordance with our results of the wound edges has been observed by Mansbridge & Knapp (1987).

Moreover it has been reported that the basal cell layer of the new epithelium showed a strong positive staining (Betz et al., 1993). Thus these differences may be due to different models or different kinds of injuries.

In this study, we have found that the cytokeratin was expressed in normal basal keratinocyte both in the regenerating epidermis and in the remaining epidermis at the wound margin. And both in the epidermis remaining at the wound margin and in the newly formed epidermis, thickening of the granular and spinous layers were observed. It may be due to the morphological changes in keratinocyte differentiation are associated with changes in cytokeratin expression.

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<국문초록>

상처회복은 염증, 재상피화 그리고 기질의 재형성등이 관여하는 복잡한 과정이다. 이중 재상피화에 관여하는 각질화세포의 이동경로를 분석하기 위하여 무당개구리 피부 상처유도 후 투과전자현미경과 cytokeratin에 대한 조직면역화학법을 이용하였다. 정상조직의 cytokeratin발현은 기저층의 세포들과 선상피에서 확인되었다.

상처 유도후 3시간 조직에서, 기저세포층에서 강한 반응이 관찰되었고, 1일과 2일 사이에서는 재생되는 각질화세포에서 강한 면역반응이 확인되었다. 상처반응 중기인 7일부터 10일 사이에서도 재생된 세포의 기저층세포에서 강한 반응이 일어났다. 19일경과의 조직에서는 기저층과 유극층의 세포들에서 cytokeratin의 발현이 증가하였다. 따라서, 재상피화에 관여하는 각질화세포는 기저층의 세포로부터 시작하여 상처부위로 이동하여 과립층과 유극층으로 분화됨으로서 재상피가 진행되었다.