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Histochemical Analysis of the Cutaneous Wound Healing in the Amphibian

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양서류 피부 상처회복과정에 대한 조직화학적 분석

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ABSTRACT

The wound healing is very complex biological processing including inflammatory, reepithelialization and matrix construction. According to the biological systematic category, the ability of the healing is very different. Generally healing ability of the lower animal group has been known more excellent compared to its higher group. Therefore, lower animals have been used as the experimental model to explore the mechanism of the wound healing or repair. To verify histochemical characteristics of the wound healing, we have used skin of the frog (*Bombina orientalis*) as known common amphibian. At day 1, 10, and 16, the mucous substance was very actively synthesized and strong positive by PAS and Alcian blue (pH 2.5). Day 10 after wounding, margin of the wound was gradually strong positive by PTAH staining for detection of collagen synthesis. At 3 to 6 hour and day 23 to 27, we have found the cell division was active through the MG P staining, in which the concentration and division of DNA in nucleus was green to deep blue color.

Key words : Amphibian, Frog, Histochemistry, Wound healing

INTRODUCTION

Wound healing is a complex process that was histologically described as having 3 distinct phase; inflammation, fibroplasia and maturation, but is now recognized as being a continuous process. Healing of the skin excision wounds involves sequential process that result

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in the removal of damaged tissue and its replacement by scar tissue (Baker & Leaper, 2000).

For many years, because of strong potential for regeneration, amphibians have been used as a model system to analyze molecular characteristics of regeneration of the limbs. Moreover skin conditions to environmental situation of the amphibians are very similar to oral mucosa of the human. It is generally reported that wound healing in oral cavity is faster than healing in skin (Sciubba et al., 1978; Hakkinen et al., 2000). Several factors are thought to contribute to this difference. In our previous study, we reported that several ultrastructural differences and similarities among the amphibian skin, human skin and oral mucosa during the healing process: a faster healing than do others was observed in the frog skin (Jeong & Moon, 1999).

Although the studies of regeneration in limbs amputation and wound healing are well documented (Brockes, 1997; Altizer, 2002), the histochemical estimations of the wound healing tissues have not been elucidated. After wound tissues obtained from excision, histochemical observations were useful to characterize amphibian skin wounds that have distinctive healing properties on the basis of previous our ultrastructural analysis.

Thus, to obtain insights about the biologic healing properties in amphibian wounds, we performed the histochemical analysis using the frog skin. And this study aims to compare the levels of histochemical properties through the several staining methods.

MATERIALS AND METHODS

1. Animals

Adult frogs, *Bombina orientalis*, were collected in Wangbang Mt. at Kyungkido, maintained in aged tap water at room temperature, and fed beetle twice weekly. Pieces of 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 up to the desired stage. Regenerates were collected at varying intervals of the post-injury: 0, 1, 4, 8, 12, 24 and 48 hr; 3, 5, 8, 11 and 14 days; 1 month, respectively. Ten frogs in each group were used. About 1/3 of the sample was used for electron microscopy.

2. Histochemical Procedures

Dorsal skin of the frog was dissected in 0.74% saline (physiological saline solution) under the stereoscope microscope (Zeiss, SV6), and fixed in 4 % formaldehyde (phosphate buffer, pH 7.2) for 1 hr. Samples were dehydrated through a graded ethanol series, embedded in the Paraplast (Sherwood Medical Industries, St. Louis). The blocks were cut 6~7 μm sections by the rotary microtome (Reichert-Jung 820). The sections were stained with various specific staining such as; Hematoxylin-Eosin, Alcian blue (pH 2.5) (Mowry, 1956), Periodic acid-Shiff (PAS), Methylgreen-pyronin (MG-P), Phosphotungstic acid hematoxylin (PTAH). The observation according to Spicer (1963) and Spicer and Sun's method (Spicer & Sun, 1967) was applied to interpret the intensity of the stained tissue: the negative reaction was −, and the symbols according to the intensity were represented as ±, +, ++, +++.

3. Electron microscopic Procedures

For the transmission electron microscopy, specimens were fixed with Karnowsky fixative for 2 hr at 4°C, washed in phosphate buffer, pH 7.4 and 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 selected areas of the tissue were thin-sectioned on the Reichert Ultramicrotome. Thin sections were stained with uranyl acetate and lead citrate and viewed in a JEOL CXII electron microscope at 80 kV.

RESULTS

1. Early responses in wound tissue

At 1 day after wounding, migrating epithelial cells were formed epithelial sheet. Cytoplasm of these cells was numerous process of slender form and intercellular space was filled with fibrous substances (Figs. 1-1, 2). Day 2 after wounding, in the sheet of epithelial cells move to wound area, the cells used fibrous substances for moving as a substrate. The junctions were not formed between cell and fibrous substance (Figs. 1-3, 4). At day 3 wound area was closed by migrating epithelial cells. Blood cells were seen under the regenerating epithelial cells (Fig. 1-5). Elongated and flatten cells were seen in migrating epithelial cells to wound middle region. Cytoplasm of these cells contained amorphous keratohyalin granules and elongated nucleus (Fig. 1-6).

Normal dorsal skin of the frog was composed of stratified squamous epithelium from cuboidal basal layer. Through the PTAH staining, collagen fibers under the epithelium was appeared pink with straw color for positive reaction (Fig. 2-1). Positive reaction of MG-P was observed in the cytoplasm of the cells in immediately injury epithelium except basal layer cells (Fig. 2-2). In PAS, intensity of the staining, especially horny and clear layer, was very strong at 3 hr after wounding (Fig. 2-3). At the same time, basal layer cells were observed with deep blue color by MG-P (Fig. 2-4). At 6 hr tissues mucous substances made several layers, which was represented deep pink color by PAS (Figs. 2-5, 6) and epithelial cells near the wounds area was strong positive with MG-P (Fig. 2-7). The migrating cells in wounds area were strong reaction with MG-P and these cells consisted of the wound closure at 12 hr tissues (Fig. 2-8).

2. Middle responses in wound tissue

At day 1, the mucous substance of the wound area was strong positive PAS and Alcian blue (pH 2.5) (Fig. 3-1). In PAS the peripheral area of the wounds was strong positive, but migrated cells to wound area were medium positive. The group of the migrated cells was 3 to 4 layers. When the cells contacted each other, movement of these cells stopped. The upper layer cells among these layers were strong positive in MG-P (Fig. 3-2). After day 3, the intensity of PAS was consistent with epithelium of day 1, but mucous substances were weak (Figs. 3-3, 4, 5, 6). MG-P staining was still strong positive in the cells near the wound margin and area at day 4 (Fig. 3-7).

3. Late response in wound healing

In day 10- wound tissues, mucous substances were disappeared and dermis area under the regenerated epithelium was strong reaction with PAS and PTAH. The wound area was substituted by new collagen fibers and

Table 1. Histochemical reactions of the cutaneous during wound healing in *Bombina orientalis*

Tissue/Test \ Time		N	0H	1H	3H	6H	12H	1D	2D	3D	4D	7D	10D	13D	16D	19D	23D	27D	31D
Epidermis	PAS	++	++	++	+++	+	++	+++	+++	+++	+++	+++	+++	++	+	+	+++	+	+++
	ALB	+	+	-	+	+	+	+	±	+	++	++	+	+	±	+	+	-	+
	MGP	++	+++	±	+++	+++	+	±	±	++	++	++	±	±	±	±	+	+	+
Dermis	PAS	+	+	++	++	++	++	+	++	++	++	+	+++	++	+	+	++	+	+
	ALB	+	+	+	++	++	++	++	++	+	++	+	+	+	+	+	+	+	+
	PTAH	++	+	+	+	+	+	+	+	++	++	±	+++	+	+	+	+	+	+

- = negative reaction, + = slight reaction, + = weak reaction, ++ = medium reaction, +++ = strong reaction.
N, normal; H, hour; D, day; PAS, periodic acid schiff; ALB, Alcian-blue pH2.5; PTAH, phosphotungstic acid hematoxylin; MGP, methylgreen-pyronin.

the cells in this area were strong reaction by MG-P (Figs. 4-1, 2, 3, 4). The horny layer cells were detached from regenerated epithelial cells and showed strong positive with PAS at day 16 (Fig. 4-5). Alcian blue staining was weak in newly formed dermal area. But it was positive (Fig. 4-6). During the day 23 and 27 MG-P was weak reaction in regenerated epithelial cells (Figs. 4-7, 8).

On the basis of these results, Table 1 represents the score of each staining according to the staining intensity.

DISCUSSION

In shark and bony fishes, wound area is accumulated by mucous substance after injury (Mittal et al., 1978; Reif, 1978). Some case of *Triturus pyrrhogaster*, wounds of cornea are covered by continuing secretion of material as considered fibrin (Yamanaka & Eguchi, 1981). In case of mammalian, wound area is covered by small clumping such as clot including damaged cells, blood, and fibrin etc. (Martin, 1997).

In this experiment, intensity of PAS staining was very strong in horny and clear layer of epithelium at 3 hr after wounding. At 6 hr tissues mucous substances made several layer, which was represented deep pink color. The mucous substance filled with wound area was strong positive PAS and Alcian blue up to day 1. After day 3, the intensity of PAS was consistent with day 1 epithelium, but mucous substance was weak. Through the observation of day 10 wounds, mucous substance was disappeared and dermis under the regenerated epithelium was strong reaction with PAS but not Alcian blue. Therefore the mucous substances play a role of the first defense for bacteria and of matrix for migration of regenerating cells.

In mammals, epithelial cells in processing necrosis region showed increasing hypertrophy and activity of mitosis (Odland & Ross, 1968; Winstanley, 1976). The increasing time point of mitosis was between 18 and 21 hours in rabbit (Viziam et al., 1964) and about 31 hours after wounding in guinea pig (Christophers, 1973). Mitotic activity is high in basal area of newly formed epithelial cells, according to this the cells are induced the differentiation into epithelium consisting normal layers (Odland, 1977; Gradwohl, 1978). In Notophthalmus, tadpole of toad, and cornea of chickens (Repesh & Oberpriller, 1980; Udoh & Derby, 1982), accumulation of epithelial cells is conducted by migrating cells toward to necrotic region (Croft & Tarin, 1970).

MG-P staining has been known useful technique to detect both RNA and DNA (Kurnick, 1952). Thus to detect the mitotic activity of the cells in healing area, we used this method. From the observation of staining intensity, positive reaction was observed in the cytoplasm of the cells in immediately injury epithelium except basal layer cells. At 3 hr to 6 hr after wounding, basal layer cells were observed with deep blue color. The migrating cells in wounds area were strong reaction. This staining pattern was still observed in the migrated cells near the wound margin and area between day 1 and 4. From this findings, the initiating time of hypertrophy and mitotic activity were observed between 3 and 6 hr in the cells of near the wound area. Thus these results indicate that time points of the mitotic activity in the frog is more fast than that of the other mammalians as shown in previously studies. This leads to fast migration of regenerated cells toward wound area.

Collagen synthesis is the most important biological activity to reconstitute matrix of the collapsed dermis (Jorgensen, 2003). During all observations period, PTAH staining was positive for collagen detection. Especially, intensity of PTAH reaction was middle to high from day 3 to 10. This implicate that collagen synthesis is gradually started from early to middle healing phase and then collagen is highly synthesized between middle and late phase. Though the period of synthesis is a little different compared to the other animals (Jeong & Moon, 1997; Ruszczak, 2003), this result is coincided with several studies (Montagna et al., 1987; Nishikori et

al., 1998).

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REFERENCES

Altizer AM, Stewart SG, Albertson BK, Borgens RB: Skin flaps inhibit both the current of injury at the amputation surface and regeneration of that limb in newts. J Exp Zool 293(5) : 467 477, 2002.

Baker EA, Leaper DJ: Proteinase, their inhibitors, and cytokine profiles in acute wound fluid. Wound Rep Reg 8 : 392 398, 2000.

Brockes JP: Amphibian limb regeneration: rebuilding a complex structure. Science 276(5309) : 81 87, 1997.

Christophers E: Kinetic aspects of epidermal healing. In: Epidermal Wound Healing (eds., Maibach HI and Rovee DT). Year Book Med. Publ., Chicago, pp. 53 69, 1973.

Croft CB, Tarin D: Ultrastructural studies of wound healing in mouse skin. I. Epithelial behaviour. J Anat 105 : 63 77, 1970.

Hakkinen L, Uitto VJ, Larjava H: Cell biology of gingival wound healing. Periodontol 2000 24 : 127 152, 2000.

Gradwohl PR: The proliferation of epidermal cells in mouse ear organ culture. Arch Dermatol Res 263 : 273 281, 1978.

Jeong MJ, Moon MJ: Fine structure of the integumentary wound healing process in the spider, Pardosa astrigera. Kor J Entomol 27(1) : 29 41, 1997.

Jeong MJ, Moon MJ: Remodeling of epidermis during skin wound healing in *Bombina orientalis*. 29(2) : 201 209, 1999.

Jorgensen LN: Collagen deposition in the subcutaneous tissue during wound healing in humans: a model evaluation. APMIS Suppl 115 : 1 56, 2003.

Kurnick NB: Histological staining with methyl green pyronin. Staining Technol pp. 233 242, 1952.

Martin P: Wound healing aiming for perfect skin regeneration. Science 276 : 75 81, 1997.

Mittal AK, Rai AK, Banerjee TK: Studies on the pattern of healing of wounds in the skin of a cat fish *Heteropneustes ossilis* (Bloch) (Heteropneustidae: Pisces). Z Mikrsk Anat Forsch 91 : 270 286, 1978.

Montagna W, Carlisle KS, Brenner RM: Histological techniques for the study of granular cells during wound healing. Am J Dermatopathol 9(1) : 26 29, 1987.

Mowry RW: Alcian blue techniques for the histochemical study of acidic carbohydrates. J Histochem 4 : 407 410, 1956.

Nishikori Y, Kakizoe E, Kobayashi Y, Shimoura K, Okunishi H, Dekio S: Skin mast cells promotion of matrix remodeling in burn wound healing in mice: relevance of chymase. Arch Dermatol Res 290 : 553 560, 1998.

Odland GF: Progression of events of epidermal differentiation in wound healing. In: Biochemistry of Cutaneous Epidermal Differentiation (eds., Seiji, M. and I.A. Bernstein). Univ. Park Press Baltimore, pp. 29 48, 1977.

Odland G, Ross R: Human wound repair. I. Epidermal regeneration. J Cell Biol 39 : 135 168, 1968.

Reif WE: Wound healing in sharks. Zoomorphology, 90 : 101 111, 1978.

Repesh LA, Oberpriller JC: Ultrastructural studies on migrating epidermal cells during the wound healing stage of regeneration in the adult newt, *Notophthalmus viridescens*. Am J Anat 159 : 187 208, 1980.

Ruszczak Z: Effect of collagen matrices on dermal wound healing. Adv Drug Deliv Rev 55(12) : 1595 1611, 2003.

Sciubba J, Waterhouse J, Meyer J: A fine structural comparison of the healing of incisional wounds of mucosa and skin. J Oral Path 7 : 214 227, 1978.

Spicer SS: Histochemical differentiation of mammalian mucopolysaccharides. Ann NY Acad Sci 106 : 379 388, 1963.

Spicer SS, Sun DCH: Carbohydrate histochemistry of gastric epithelial secretions in dog. Ann. NY Acad Sci 140 : 762 783, 1967.

Udoh P, Derby A: The effect of the substrate on tadpole epidermal cells in vitro. J Exp Zool 219 : 75 80, 1982.

Viziam CB, Matoltsky AG, Mescon H: Epithelialization of small wounds. J Invest Dermatol 43 : 499 507, 1964.

Yamanaka H, Eguchi G: Regeneration of the cornea in adult newts: Overall process and behavior of epithelial cells. Differentiation 19 : 84 92, 1981.

<국문초록>

상처회복과정은 염증, 재상피화, 그리고 기질의 재형성등을 포함한 포괄적인 생물학적 반응이다. 생물학적 분류체계에서, 회복능력은 매우 다양한데, 일반적으로 하등동물이 고등동물에 비교해 단순하며 뛰어난 것으로 알려져 있다. 따라서, 하등동물들을 이용한 실험모델은 상처회복과 치유의 기작을 연구하는데 자주 이용되고 있다. 양서류를 이용한 피부 상처회복과정 동안의 조직화학적 특성들을 규명하기 위하여 한국산 무당개구리(*Bombina orientalis*)를 이용하였다. 1일, 10일 그리고 16일의 조직에서 점액물질이 활발하게 분비되는 것을 확인하였으며, PAS (periodic acid shiff)와 Alcian blue (pH 2.5) 반응에 강하게 염색되었다. PTAH (phosphotungstic acid hematoxylin) 염색을 통하여 상처 후 10일 조직에서 상처주변의 진피층에서 콜라겐의 합성이 증가하는 것을 확인하였다. 또한, 세포의 유사분열의 시점을 확인하기 위한 MG-P (methyl green pyronin)염색에서 초기에는 3시간과 6시간에 강한 염색반응을 관찰하였고, 이는 23일과 27일 조직에서도 동일한 패턴으로 이어졌다.

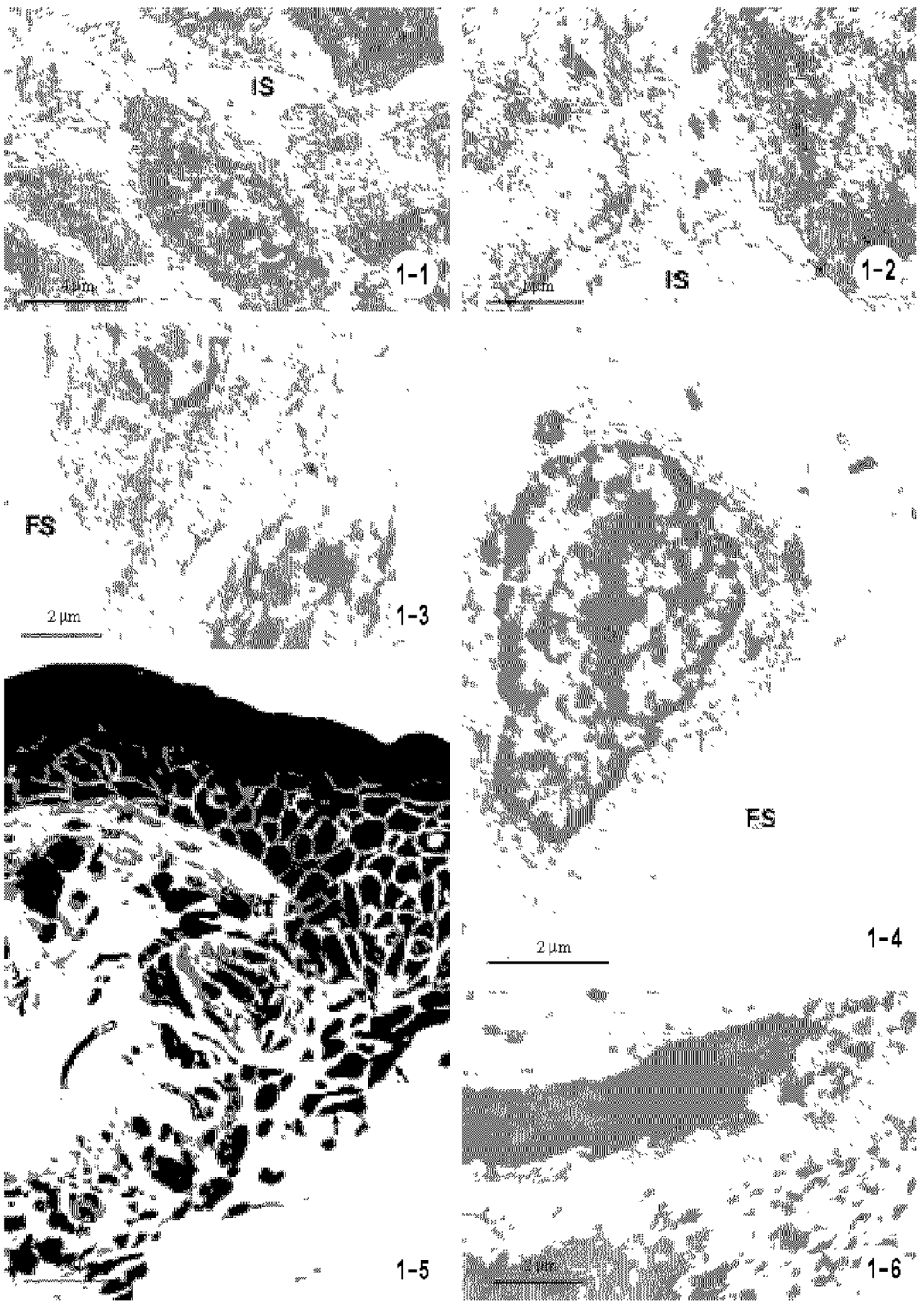
FIGURE LEGENDS

Fig. 1. Fine structural demonstration in the early healing responses.
1, 2. At day 1 after wounding, the migrating epithelial cells were slender form and intercellular space (IS) was filled with fibrous substances. 3, 4. At day 2 after wounding, sheet of epithelial cells moved to wound area by fibrous substances (FS). 5. Regenerating dorsal skin at day 3. Arrows indicate direction of regenerating epithelial cells. 6. Elongated and flatten cells were seen in migrating epithelial cells to middle region of the wound and contained amorphous keratohyalin granules and elongated nucleus.

Fig. 2. Early responses in wound tissues.
1. Normal skin tissue (Phosphotungstic acid hematoxylin). The collagen fibers under the epidermis appeared as a brownish pink color with the PTAH staining method. 2. After injury, the wounded skin showed a retraction of damaged surface (Methyl-green pyronin). 3. PAS staining at 3 hours after wounding. The degree of positive reaction to wound marginal epithelial tissue varied from horny layer to basal layer. The horny layer and clear layer were strong positive reaction. 4. At 3 hours after wounding, the cells in wound edge were stained positive with the MG-P staining. Especially, basal layer cells in epithelium were deep blue color. 5, 6, 7. The mucous substance and wound marginal tissue were stained strong reddish purple to purple at 6 hours. Epithelial cells were located wound margin and edge which are strong positive in MG-P staining. Cytoplasm of the cells in wound margin is stained purple to pink color. 8. At 12 hours, epithelial cells spread over the wound surface by migration. MG-P staining reaction appeared strong positive in migrating cells. It has been observed that all kinds of cells near the wounds seem to have the ability to participate in migration.

Fig. 3. Middle responses in wound tissues.
1. At day 1 post-wounding, mucous layer was much increased in thickness and showed strong positive in PAS and Alcian blue pH2.5 staining. 2. After day 3, regenerated epithelial layer consisted of four or five cell layers which were similar to horny, clear, granular, and spinous layer. The horny and clear layer was strong pink color in MG-P staining. The granular and spinous layer were much less color than clear and horny layer. 3, 4, 5, 6. Growth and differentiation of the epithelial cells were increased at 4 days. The degree of positive reaction was equal to 3 days tissue. Mucous layer shows week positive reaction in PAS. The regenerating epithelial layer is stained blue color in upper region by Alcian blue staining. 7. At day 4, the epithelial cells during growth and differentiation, were stained deep blue and sky blue in MG-P.

Fig. 4. Late responses in wound tissues.
1, 2, 3, 4. At day 10 after injury, intercellular collagenous fibers appeared along the cellular reticular fibers. Marginal wounded dermis is strong positive in PAS and PTAH staining. The regenerating epithelial layer was equal to normal epidermis containing five layers. A prominent feature at this time, mucous substances were not observed. Wound dermis was substituted mucous substances for collagenous fibers and ground substances. The cells located in this region were strong positive in MG-P. 5, 6. At day of 16th, keratinised layer was shed and replaced by the progressive movement and maturation of cells from the basal layer in regenerating epithelial layer and PAS reaction was strong. This area was week positive in Alcian blue. 7, 8. At 23 and 27 days, collagen fibers were increased and numerous connective tissue cells appear. Epidermis of regenerated tissue was stained strong color in MG-P.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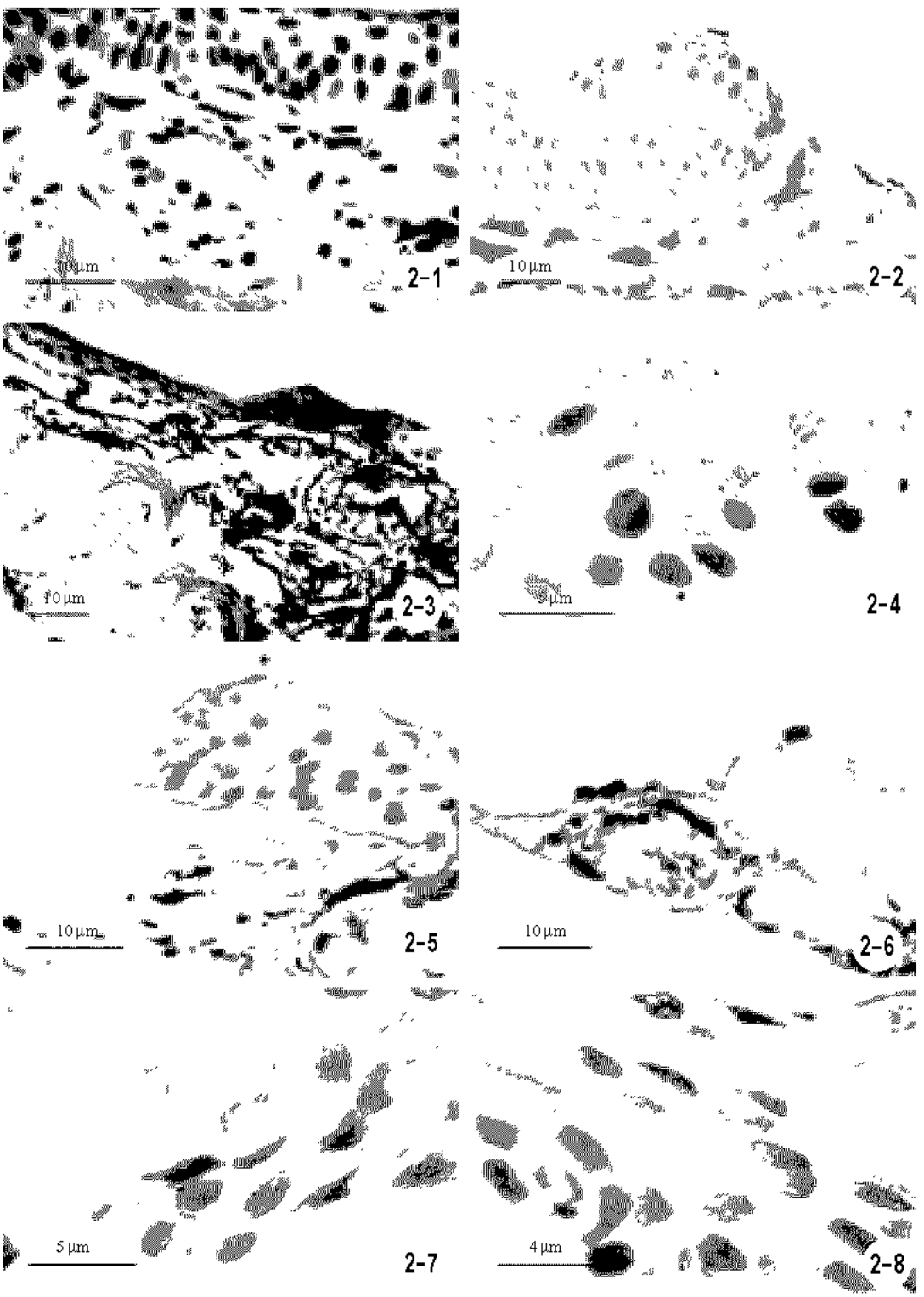
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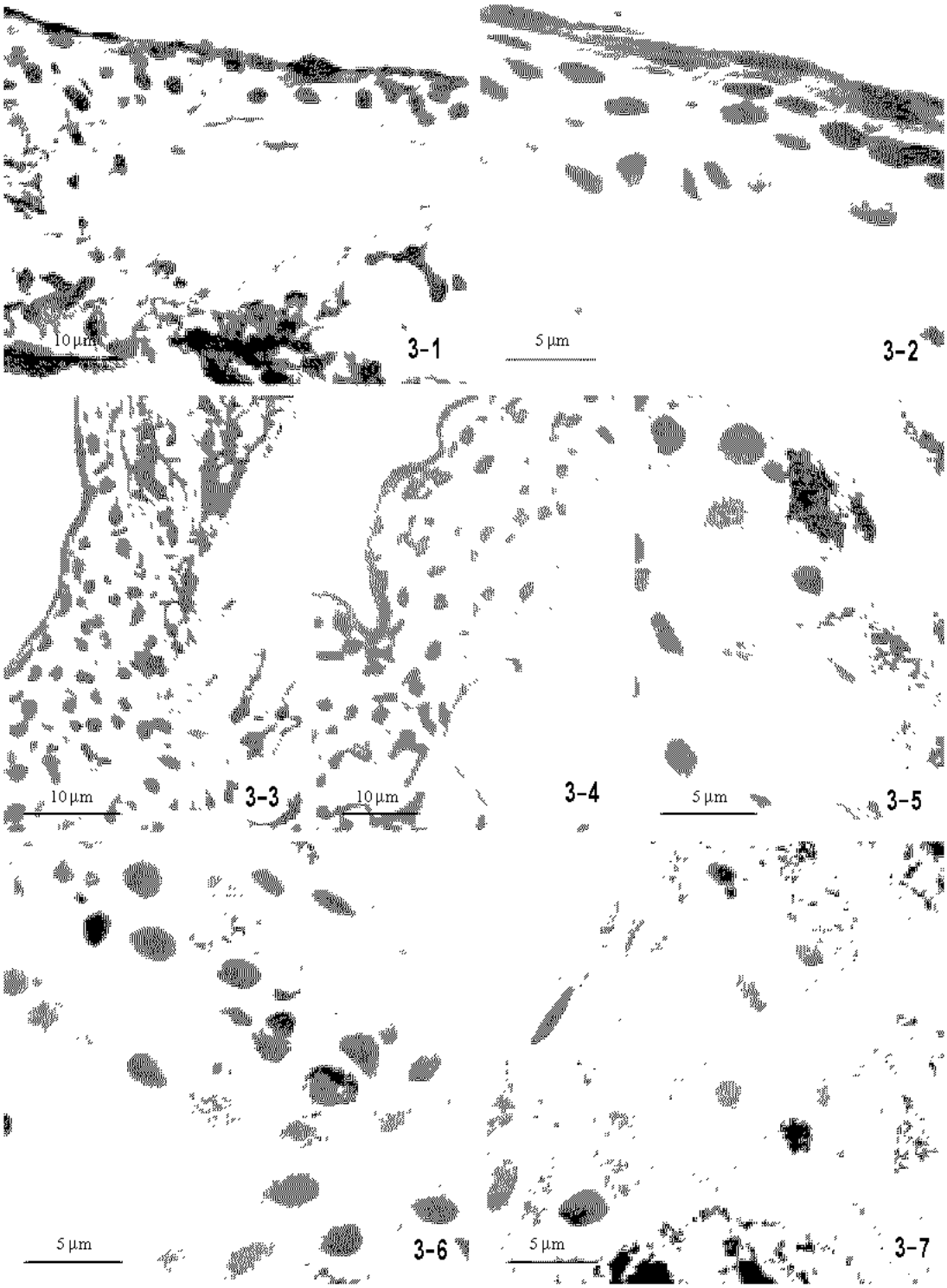
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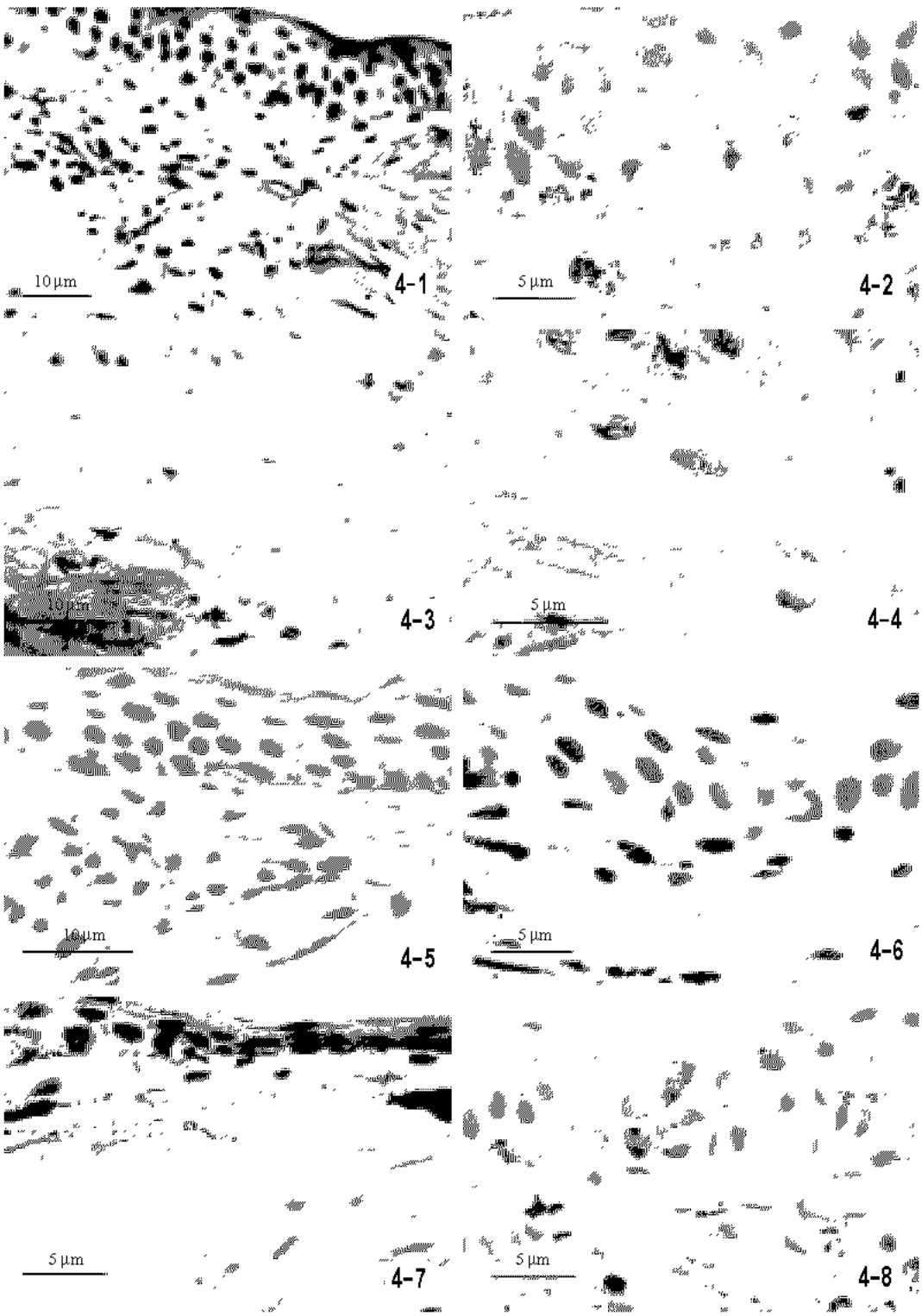
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