한국전자현미경학회지 제 32 권 제 3 호 2002
Korean J. Electron Microscopy 32(3), 275~284(2002)

Fine Structural Approach of Granular Gland Regeneration after Skin Injury in *Bombina orientalis*

Moon-Jin Jeong*, Do-Seon Lim[1] and Myung-Jin Moon[2]
Cellular Immunology Section, Oral Infection & Immunity Branch, National Institute of Dental & Craniofacial Research (NIDCR), NIH, Bethesda, MD, 20892-4352, USA
[1]Department of Dental Hygiene, Seoul Health College, Sungnam, 461-713, Korea
[2]Department of Biological Sciences, Dankook University, Cheonan, 330-714, Korea
(Received August 1, 2002; Accepted August 31, 2002)

Bombina orientalis 피부손상 후 과립선 재생에 관한 미세구조적 연구

정 문 진*, 임 도 선[1], 문 명 진[2]
미국 국립보건원 치구개안면연구소 구강감염 및 면역부 세포면역연구실
[1]서울보건대학 치위생과, [2]단국대학교 생물학과

ABSTRACT

Granular gland regeneration in the toad after dorsal skin wound histologically was examined using scanning and transmission electron microscopy. After cutaneous wounds were induced by excision, animals were maintained in special cages for up to 20 days. In transmission electron microscopy (TEM), newly formed granular gland, though poorly developed, was seen on 4 day after injury. Epithelial cells moved toward apical region of newly formed gland. The cells had smooth surface and were not connected to other cells by desmosomes. Mitochondria rich cell (MRC) possessing long cytoplasmic processes formed a gland cavity and hemidesmosomes were found under the cell processes. Basal cavity of newly formed gland consisted of MRC, pro granular producing cells (pGPC), and granular producing cell (GPC). Moreover it was observed that xanthophores moved to the base of the epithelial tissue on 10 day after the injury. These cells contained numerous pterinosomes and carotenoid vesicles. Immature pterinosomes were large and carotenoid vesicles were moderately electron dense. On 13 day after the injury, xanthophores contained abundant carotinoid vesicles and lammelated pterinosomes. Iridophores were also observed adjacent the developing xanthophores on 16 day post injury. These observations indicated that regeneration of granular gland from glandular precursor cells during wound healing and subsequent expansion of the glandular cells might be dependent on maturation and proliferation of these newly formed cells.

Key words : Granular gland, Pigment cells, Regeneration, Skin injury

This work was supported by Grant from the Ministry of Education.
* Correspondence should be addressed to Dr. Moon-Jin Jeong, Cellular Immunology Section, Oral Infection & Immunity Branch, National Institute of Dental & Craniofacial Research (NIDCR), NIH, Bethesda, MD, 20892-4352, USA. Ph.: 1-301-402-4046, FAX: 1-301-402-1064, E-mail: jeongmj4@hanmail.net

INTRODUCTION

Re-epithelialization results from epithelial cells (keratinocyte) migration and proliferation. In superficial skin wounds, the basement membrane is not damaged and re-epithelialization is a fast and efficient process (Clark, 1998). However, when basement membrane is destroyed, as in deep partial or full-thickness wounds, re-epithelialization is slow and occurs onto a provisional extracellular matrix that changes during formation of granulation tissue and remodeling of the wound continues for long time until a scar is formed (Clark, 1988; Juhasz et al., 1993).

Hair follicles have long been considered to be the primary source of these appendageal keratinocytes; a role for eccrine or apocrine structure has only rarely been suggested (Lobitz et al., 1954). The keratinocytes become activated and initiate repair of epidermal wounds along lateral margins of a wound as well as in residual appendageal structures that remain at its base (Al-Barwari & Potten, 1976; Argyris, 1976). The keratinocytes in salivary glands display characteristics of epithelia (Leeson & Leeson, 1971) and are thought to play an important role in the histogenesis of several kinds of salivary gland tumors (Morinaga et al., 1987). It is clinically important to understand regenerative processes in salivary glands. Several investigators have examined the regeneration of ducts and acini in partially injured salivary gland (Morimura, 1988; Takahashi & Wakita, 1993, 1994), but few have considered the regeneration of granular glands.

With regard to glands regeneration, published work on wound healing is limited to human and rat glands such as sweat glands (Miller et al., 1998), salivary glands (Leeson & Leeson, 1971) and acinar glands (Takahashi et al., 1998). Only limited data exist regarding the regeneration of granular glands during wound healing processes, especially, in lower animal like toads. In this study we focused on the regeneration in which granular gland might function as a potential source of keratinocyte for re-epithelializing wounds and other glandular precursor cells. Since it was necessary to eliminate the gland epithelium as a source of keratinocyte, we created partial thickness wounds sufficiently deep to completely remove the entire granular glands. Since the present results were obtained after full-thickness wounding (epithelial and dermal tissue), it was also of interest to determine whether impaired gland could be repaired. Further, it is not clear which cell types contribute to the reconstruction of glands after injury.

In the present study, therefore, we investigated cellular events such as cell migration, regeneration of gland composed of various cell types, and regeneration of dermal chromatophores. For this purpose, light microscopic and transmission electron microscopic analysis were employed at various times after mechanical cutaneous wounds. From our results it was found that glandular precursor cells first arose on day 4 and subsequent growth might be largely dependent on the progressive differentiation and proliferation of these newly formed cells.

MATERIAL AND METHODS

Adult toads, *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. Toads were then returned to water and allowed to regenerate to the desired stage. Regenerates were collected at varying intervals after injury: 0, 1, 4, 8, 12, 24 and 48 hr; 3, 5, 8, 11, and 14 days; 1 month, respectively. Ten toads in each group were used. About 3/5 of the sample was used for transmission electron microscopy and 2/5 for histochemistry.

For optical microscopy, each wound, also including surrounding uninjured skin, was excised and fixed in

10% neutral buffered formalin, pH 7.2. Samples were dehydrated through a graded ethanol series and embedded in Paraplast (Polyscience, USA). From each Paraplast-embedded strip one 5 μm section was cut, deparaffinized in xylene, dehydrated in a descending alcohol series and stained with haematoxylin-eosin.

To identify cellular fine structures we used transmission electron microscopy. Specimens were subjected to a double fixation. First they were fixed with Karnowsky fixative (1967) 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 (Polyscience). Semi thin (1 μm) sections were stained with toluidine blue (1% borax) and viewed in the photomicroscope (Olympus CH30, Japan). Selected areas of the embedded tissue were thin sectioned on Reichert Ultramicrotome (LKB Co., Sweden), stained with uranyl acetate and lead citrate (Reynolds, 1963) and observed in a JEOL CXII (JEOL, Japan) electron microscope at 80 kV.

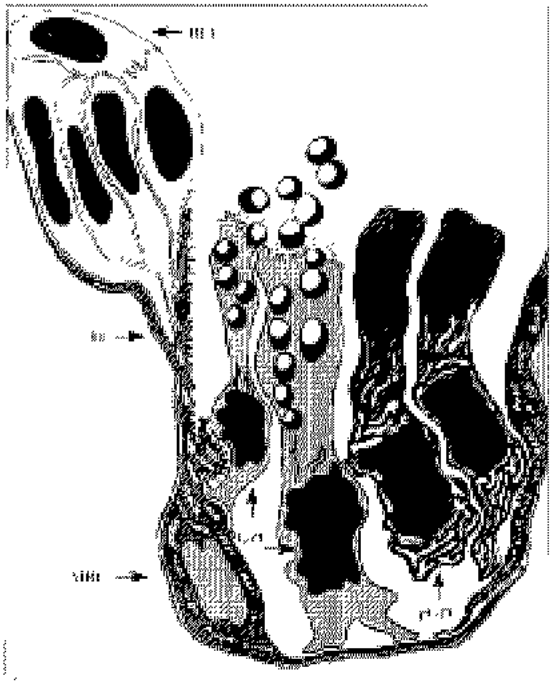

Fig. 1. Diagrammatic illustration of granular gland formation during skin wound healing responses. BL, basal lamina; REC, regenerating epithelial cell; MRC, mitochondria rich cell; GPC, granule producing cell; pGPC, pro-granule producing cell.

RESULTS

1. Identification of three types of cells in regenerating glands

To examine whether the gland was regenerated in skin healing process, we used transmission electron microscopy for observation of morphological cellular event of the injured gland. On 4 day after injury, it was observed that gland was regenerated in wounded skin and secretion of granules occurred by granule producing cell (GPC) (Fig. 2-1). We also identified four types of cells participated in gland formation during healing process; epithelial cells (EC), mitochondria rich cells (MRC), pro-granule producing cells (pGPC), and granule producing cells (GPC) (Fig. 1; Fig. 2-3). These cells were clearly distinguished by fine structural characteristics.

Part of epithelial cells participated in regenerating skin moved to region of dermis. These cells were in spindle shape and kept a distance at a uniform intercellular space. Front region of the cells had long and slender cell processes. MRC observed in basal cavity of the gland made long cytoplasmic processes to migrating epithelial cells. Cell processes of some epithelial cells made cellular contact with MRC cytoplasmic processes (Fig. 2-2, 3).

MRC first appeared in injured dermis at one day after wounding. They were astrocyte-like in shape with large and long cytoplasmic processes. These cells contained numerous rod type mitochondria and glycogen granules in their cytoplasm and phagocytic vacuoles including dead cell were observed (Fig. 2-4). Some of MRC were

localized in along plywood-like collagen fibers. These cells protected damaged dermis by elongated cytoplasmic processes. Basement membrane on basal area of the cells was also observed. In addition to the evidence of defense, the structures revealing phagocytic activity were seen in Fig. 2-4, 5.

pGPC and GPC were localized in basal area of the gland and contacted with cytoplasmic process of MRC (Fig. 3-1, 2). Nucleus of pGPC was located in basal part of the cells and elongated cytoplasmic processes were observed in apical part. These cells contained well-developed rough endoplasmic reticulum, Golgi and mitochondria near the nucleus (Fig. 3-3, 4, 5). In these cells, some cytoplasmic processes with well-developed microvilli structure were observed (data not shown).

GPC contained secretory granules, electron dense materials, and well-developed rough endoplasmic reticulum near the nucleus. In apical region of the cells, granules containing part of cytoplasm were secreted (Fig. 1; Fig. 3-3, 6, 7).

2. Rearrangement of pigment and endothelial cells

Newly formed collagen fibers in regenerating dermal area were observed on 10 day after wounding. At this time point, xanthophores were localized under the regenerated epithelium (Fig. 4-1). The xanthophores became elongated in shape and contained irregular shaped nucleus. Pterinosome, carotenoid, and small vesicles were distributed throughout the entire cytoplasm. Pterinosome was an oval or a globular in shape with well-developed or developing lamella structure. Carotenoid vesicles were low electron dense and observed together with other small vesicles (Fig. 4-2). Some cells had numerous rER, which were observed around these chromophores (Fig. 4-3, 4).

In regenerated dermal region on 13 day, the size of the xanthophores was larger than those on 10 day. These cells contained very invaginated and lobuled nucleus. Moreover, the number of pterinosome and carotenoid vesicles was significantly increased (Fig. 4-5). Iridophores (after 16 day), appeared later than xanthophores, were localized under the basement membrane of regenerated epithelial cells and elongated in shape (Fig. 4-5). Reflecting plates, consisted of limiting membrane, were observed in their cytoplasm. At this time, iridophores were also observed under the xanthophores (Fig. 4-6).

On 19 day after wounding, early-observed melanophores moved across the collagen fibers. Most of these cells were found around iridophores. High electron dense granules accumulated in their cytoplasm and nucleus were located at the peripheral membrane area (Fig. 4-7, 8). Formation of vascular system was detected on 19 day after wounding. At this time, endothelial cells were extensively lobuled and elongated in shape. One endothelial cell process made cellular contact with the others. However, in this cellular contact, any cell binding structures such as desmosome were not observed (data not shown).

DISCUSSION

In mammals and birds regeneration of cutaneous tissue is restricted to a few examples. For instance, no hairs and glands are developed in the area of a wound after healing, even in embryonic chick skin no feather buds develop after wounding (Thevenet, 1983). However, rabbit ears are unique in that they are the only ears known to close and fill in holes which have been punched through them (Goss & Grimes, 1975). Recently, glands formation is reported in submandibular region in rat after laser irradiation (Takahashi et al., 1997).

The findings in the present study showed that the amphibian skin regenerates epidermal construction such as glands, chromatophores, and vascular system throughout the healing process. On 4 day after injury, keratinocytes were concerned in duct regeneration of gland. Epithelial cells participated in regeneration of skin

moved to region of dermis and were in spindle shape with keeping a distance at a uniform intercellular space. The cells of front region had long and slender cell processes and led to regeneration of duct. According to previous reports, it has been known that gland morphology returns to normal after duct recanalization (Tamarin, 1971a; Takahashi et al., 1998). Therefore it was assumed that formation of duct by epithelial cells is the first step of gland regeneration.

In the present study, the pattern of cell migration during gland formation was observed. Movement of regenerative epithelial cells into middle region was shown in cell-free face generated by artificial wounding and the pattern of rolling was observed in these cells. This finding is coincided with our previous report (Jeong & Moon, 1998). We also observed other cells with the pattern of sliding movement. These epithelial cells moved to cytoplasmic processes of MRC. MRCs in injured dermis were astrocyte-like in cell shape since they possessed large and long cytoplasmic processes. The cells had smooth surface and were not connected to other cells by desmosomes. Constant space was observed between cells. These cells protected damaged dermis by elongated cytoplasmic processes. Basement membrane on basal area of the cells was also observed. Cytoplasm of the cells contacted with each other and epithelial cells stopped growth by contact inhibition. From this observation, it is implied that amphibian epithelial cells have a pattern of identical movement compared with mammalian cells (especially epithelial cells) in wound healing process.

The present study demonstrates that four types of cells are involved in regeneration of gland: epithelial cells, mitochondria rich cells, pro-granule producing cells, and granule producing cells (shown in Fig. 1; Fig. 3-3). In case of rat parotid duct, complete obstruction of healing results in loss of all acinar cells (Walker & Gobe, 1987). This finding was made by the significant loss in gland weight and failure to identify acinar cells ultrastructurally after complete obstruction (Redman, 1994). Therefore these reports indicate that residual epithelial cells and duct cells were not dedifferentiated acinar cells. In toads, glandular regeneration was completed more slowly than those in other studies (Burford-Mason et al., 1993; Cummins et al., 1994). Duct cell proliferation in the duct-ligated salivary gland is accomplished more fast than in an area of total glandular destruction, where duct have to be reconstituted, before significant acinar cell differentiation and proliferation can proceed (Takahashi & Wakita, 1993). In exocrine glandular tissue, as a whole, stem or precursor cells are thought to reside in ducts.

In contrast to our findings, it has been reported granular cell can synthesize granules in the absence of an efferent ductal pathway (Tamarin, 1971b). After this pathway is reestablished, the synthesis of more granules is associated with reconstruction and increase in the amount of rER and Golgi areas. However, GPCs and pGPCs in the early phase are quite different from those in normal granular cells since they appear dense. In conclusion, our finding suggests that remodeling of secretary gland may give rise to precursor cells through ongoing differentiation and proliferation.

ACKNOWLEDGEMENT

We thank to Dr. Sang-Seop Lee at NIDDK, NIH for critical reading of the manuscript. This work was supported by grant from the Ministry of Education.

REFERENCES

Al Barwari SE, Potten CS: Regeneration and dose response characteristics of irradiated mouse dorsal epidermal cells. Int J Radiat Biol Relat Stud Phys Chem Med 30(3) : 201 216, 1976.

Argyris T: Kinetics of epidermal production during epidermal regeneration following abrasion in mice. Am J Pathol. 83 (2) : 329 340, 1976.

Burford Mason AP, Cummins MM, Brown DH, Macan A, Dardick I: Immunohistochemical analysis of the proliferative capacity of duct and acinar cells during ligated induced atrophy and subsequent regeneration of rat parotid gland. J Oral Pathol Med 22 : 440 446, 1993.

Clark AJ: The mammary gland as a bioreactor: expression, processing, and production of recombinant proteins. J Mammary Gland Biol Neoplasia 3(3) : 337 350, 1998.

Clark AJ: Ectopic hormone production. Baillieres Clin Endocrinol Metab 2(4) : 967 986, 1988.

Cummins M, Dardick I, Brown D, Burford Mason A: Obstructive sialadenitis: A rat model. J Otolaryngology 32 : 50 56, 1994.

Goss RJ, Grimes LN: Epidermal down growths in regenerating rabbit ear holes. J Morphol 146(4) : 533 542, 1975.

Jeong MJ, Moon MJ: Early healing responses of the skin wounds in the korean fire bellied toad, *Bombina orientalis*. Kor J Electron Micros 28(3) : 329 343, 1998.

Juhasz I, Murphy GF, Yan HC, Herlyn M, Albelda SM: Regulation of extracellular matrix proteins and integrin cell substratum adhesion receptors on epithelium during cutaneous human wound healing in vivo. Am J Pathol 143(5) : 1458 1469, 1993.

Karnovsky MJ: The ultrastructural basis of capillary permeability studied with peroxidase as a tracer. J Cell Biol 35 : 213 236, 1967.

Leeson TS, Leeson CR: Myoepithelial cells in the exorbital lacrimal and parotid glands of the rat in frozen etched replicas. Am J Anat 132(2) : 133 145, 1971.

Lobitz WC, Holyoke JB, Montagna W: Responses of the human ecrine sweat duct to controlled injury. J Invest Dermatol 23 : 329 344, 1954.

Miller SJ, Burke EM, Rader MD, Coulombe PA, Lavker RM: Re epithelialization of porcine skin by the sweat apparatus. J Invest Dermatol 110 : 13 19, 1998.

Morimura A: Immunohistochemical study on regeneration of the rat submandibular gland. Shikwa Gakuho 88 : 511 528, 1988.

Morinaga S, Nakajima T, Shimosato Y: Normal and neoplastic myoepithelial cells in salivary glands: an immunohistochemical study. Hum Pathol 18(12) : 1218 1226, 1987.

Redman RS: Myoepithelium of salivary glands. Micros Res Techni 27 : 25 45, 1994.

Takahashi S, Wakita M: Regeneration of the lobular duct and acinus in rat submandibular glands after laser irradiation. Arch Histol Cytol 56 : 199 206, 1993.

Takahashi S, Wakita M: Cytokeratin expression during regeneration of the intralobular duct in rat submandibular glands after YAG laser irradiation. Arch Histol Cytol 57(2) : 167 173, 1994.

Takahashi S, Domon T, Yamamoto T, Wakita M: Regeneration of myoepithelial cells in rat submandibular glands after yttrium aluminium garnett laser irradiation. J Exp Path 78 : 91 99, 1997.

Takahashi S, Schoch E, Walker NI: Origin of acinar cell regeneration after atrophy of the rat parotid induced by duct obstruction. J Exp Path 79 : 293 301, 1998.

Tamarin A: Submaxillary gland recovery from obstrction. II. Electron microscopic alterations of acinar cells, J Ultrast Res 34 : 288 302, 1971a.

Tamarin A: Submaxillary gland recovery from obstruction. Overall changes and electron microscopic alteration of glandular duct cells. J Ultrastruc Res 34 : 276 287, 1971b.

Thevenet A: Cicatrisation de la peau d'embryon de 7 jours cultivee in vitro. Arch Microsc Morphol Exp 72 : 23 46, 1983.

Walker NI, Gobe GC: Cell death and cell proliferation during atrophy of the rat parotid gland induced by duct obstruction. J Pathol 153 : 333 344, 1987.

<국문초록>

두꺼비 등 피부손상 후 과립선의 재생과정을 주사전자현미경과 투과전자현미경으로 관찰하였다. 절개에 의하여 피부손상을 가한 후 실험을 위하여 특수히 제작된 cage 내에서 최대 20일간 사육하였다. 투과전자현미경 관찰에서 손상 4일 후 미성숙 형태의 신생 과립선이 관찰되었으며, 상피세포는 신생 과립선의 첨단부로 이동하여 있었다. 상피세포의 표면은 편평하였으며 desmosome에 의해 서로 연결되어 있지 않았다. 미토콘드리아를 많이 함유한 세포돌기 (MRC)들이 선의 내강을 형성하고 있었고, 이들 돌기에서는 hemidesmosome이 관찰되었다. 신생선의 기저강은 MRC, 과립형성전세포 및 과립형성세포 등으로 이루어져 있었다. 특히, 손상 후 10일에 xanthophore가 상피세포의 기저부로 이동하여 있음이 관찰되었다. 이들 세포는 다수의 크기가 큰 pterinosome과 중등도의 전자밀도를 가진 carotenoid vesicle을 포함

하고 있었다. 손상 후 13일에, xanthophore는 많은 carotinoid vesicle과 lammelated pterinosomes을 포함하고 있었다. Iridophore는 손상 16일에 분화중인 xanthophore 주변에서 관찰되었다. 이러한 소견은 손상으로부터의 회복 과정에 선조세포(glandular precursor cell)로부터 과립선이 재생되며, 선세포의 팽대는 이들 신생 세포의 성숙 및 증식에 의한 것을 의미한다.

FIGURE LEGENDS

Fig. 2. Fine structural views of duct and MRC in newly formed granular gland.

1. TEM image of secretion of granules (G) through duct of gland. Gland is newly formed after wound closure on 4 day after injury.
2, 3. Epithelial cells of duct region in gland. These cells take a sliding movement. The cells have smooth surface but are not connected to other cells by desmosomes. Constant space is observed between cells. Long and slender cellular processes (CP) are observed in front region of the cells.
4, 5. Phagocytosed dead cells are observed in cytoplasm of mitochondria rich cell (MRC). It is observed that damaged dermis after one-day post injury. Enlarged cytoplasm contains abundant rod type mitochondria (Mt).

Fig. 3. Regenerating cells in cavity and bottom region of gland.

1, 2. Long process of mitochondria rich cell forms a gland cavity. Hemidesmosomes (Hd) are formed under the cell process.
3. Basal cavity of newly formed gland consists of mitochondria rich cell (MRC), pro granular producing cell (pGPC), and granular producing cell (GPC).
4. TEM images of pro granular producing cell. rER are abundant in cytoplasm. Granular producing cell is seen below this cell.
5. Higher magnification of well-developed rER in the granular producing cell. Golgi apparatus (GA) is observed in the cytoplasm.
6. Pro granular producing cell is irregular in shape with long cytoplasmic processes. These processes were in contact with processes of adjacent mitochondria rich cell (arrow).
7. Higher magnification of elongated cell process of pro-granular producing cell. The cell junctions are not formed between two cell processes (circle).

Fig. 4. Regenerating and arrangement of dermal pigment cells under the epithelial cell layers.

1, 2, 3. It is observed that xanthophores (Xn) move to under the epithelial tissue on 10 day after wounding. These cells contain numerous pterinosomes (Pt) and carotenoid vesicles (CV). Immature pterinosomes are large and carotenoid vesicles moderately electron dense.
4. The cell containing abundant rER in cytoplasm is found near the chromatophores.
5. On 13 day after wounding, xanthophores contain abundant carotinoid vesicles and lammelated pterinosomes. Mitochondria are found near the lobulated nucleus.
6. Iridophores (Ir) are observed adjacent the developing xanthophores on 16 day post injury. Cytoplasm of these cells contains numerous reflecting platelets (RP).
7. Photomicrograph of regenerating skin on 19 day post injury. Arrows indicate regenerated melanophores.
8. Melanophores (Me) including numerous melanosomes in cytoplasm are observed under the iridophores.

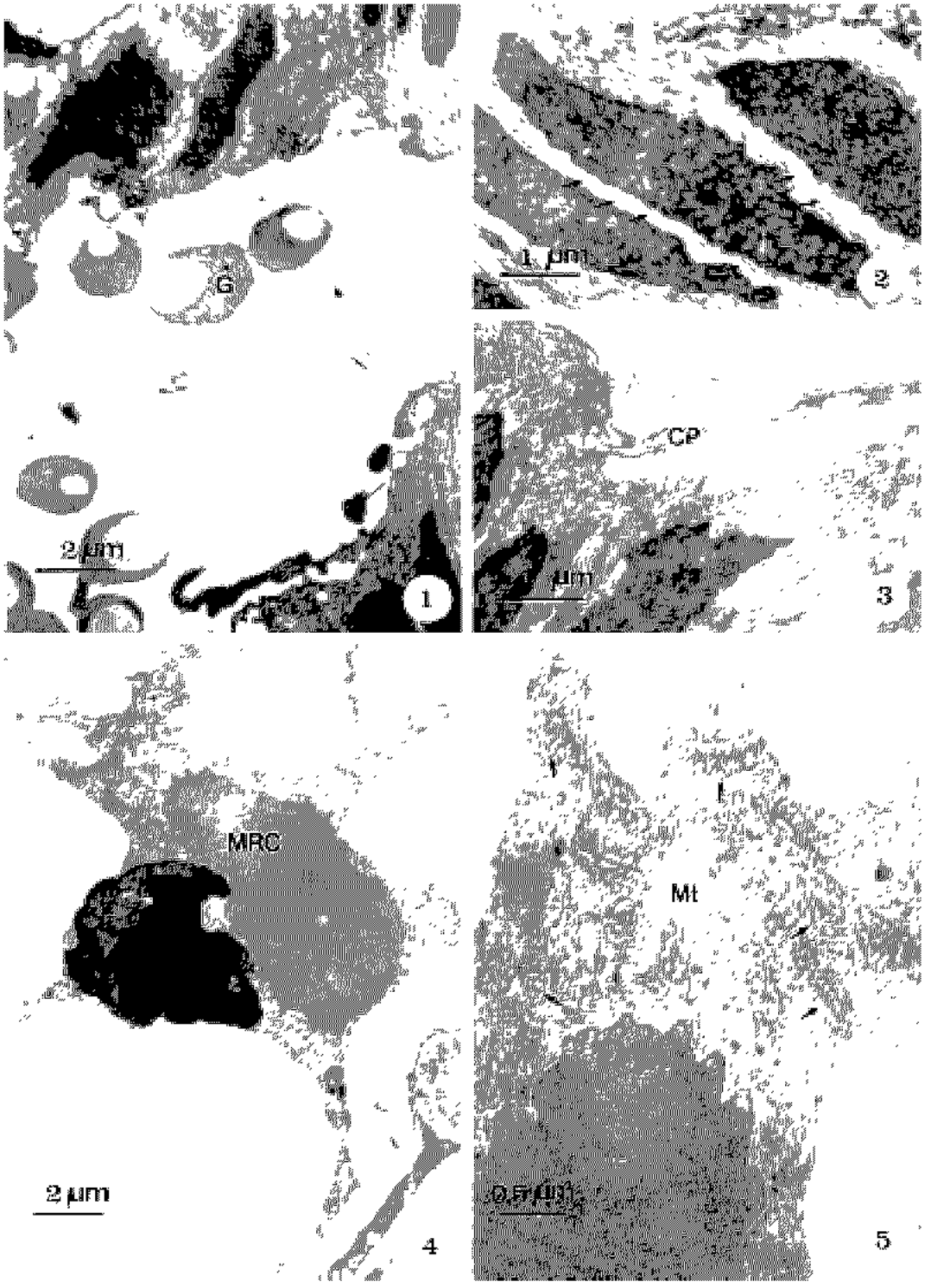

Fig. 2

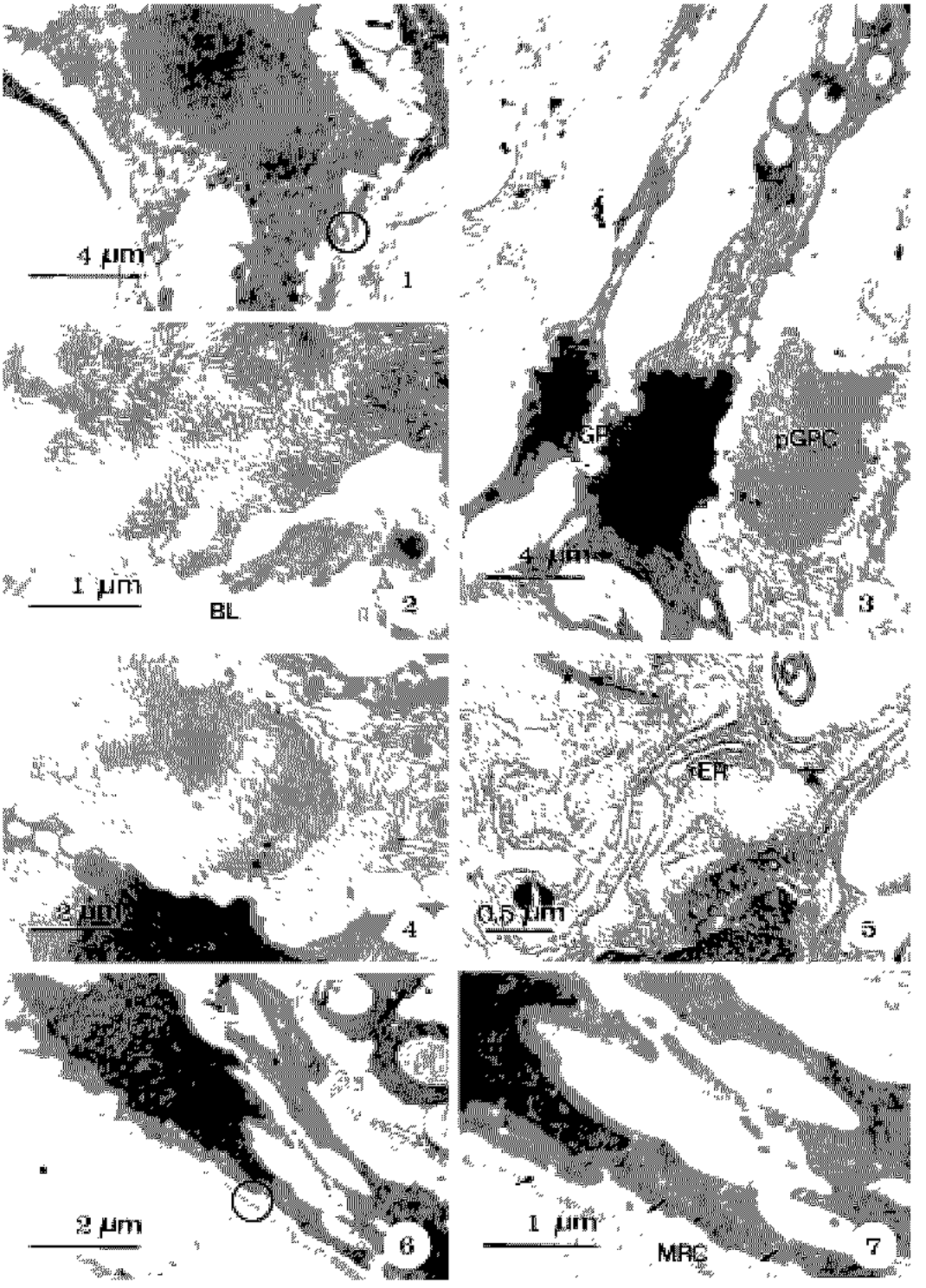

Fig. 3

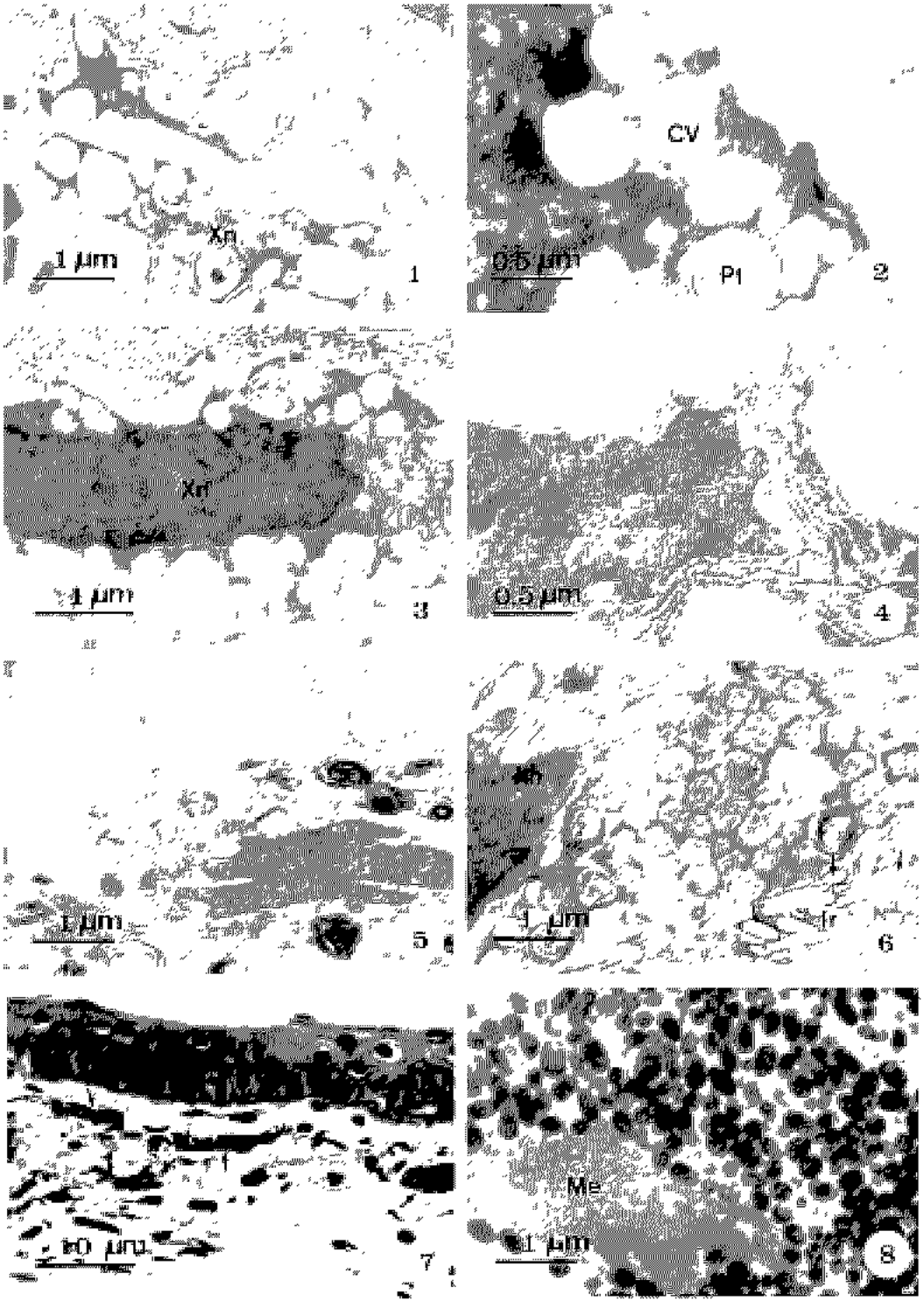

Fig. 4