Cell Patterns in Open Wound Healing

—Light and Electron Microscopic Observations—

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ABSTRACT

Cell patterns in open wound healing are studied by both light and electron microscopic examinations in regards to time sequence, metamorphosis, and functional aspects.

Process of the open wound healing clearly exhibited not only time sequence of cellular appearance but also zonation of cells. In the initial stage, until the 3rd day, the neutrophilic polymorphonuclear leukocytes were predominant and particularly concentrated in the scab region.

The mononuclear cells were active cells during the 1st to 7th day and were mainly concentrated in the subscab region. The fibroblastic activities started from the 3rd day and became very active during the 5th to the 10th day, and they were concentrated at granulation tissue region.

During the process of wound healing, the cellular elements underwent metamorphosis; The neutrophils from normal to swollen and finally degenerating; the mononuclear to macrophages; the fibroblasts from immature to mature actively protein synthesizing cells.

The functions of each cellular element can not be determined with certainty. However, the main function of neutrophils in wound healing is likely the formation of front line defense as a part of the scab formation on the surface. And the major function of mononuclear cells is to debride exudates and damaged tissue debris especially at the subscab area and that of the fibroblasts to replace the tissue defect by proliferation and production of fibrous proteins.

INTRODUCTION

Numerous investigations have been made on wound healing in regards to cellular elements, extracellular elements, and various factors that modify the process of wound healing. The major cellular elements concerned with wound healing include segmented neutrophilic granulocytes, macrophages, fibroblasts, and endothelial cells (Ross and Benditt 1961, Ross and Odland 1968, Gillman 1968, Ross 1968).

Simpson and Ross (1972) studied the role of neutrophilic leukocytes in wound healing by observing the progress of repair in the absence of these cells. They concluded that neither wound debridement nor the formation of granulation tissue are dependent upon the presence of neutrophils. Leibovich and Ross (1975) studied the role of macrophages in wound repair and stated that the macrophage is the principal cell type responsible for wound debridement, and also the macrophage may stimulate fibroblastic proliferation. Replacement of damaged tissue by fibrous tissue is the ultimate process of wound healing. However, the mechanism and process of fibroblastic
activation is still uncertain.

Present study is to investigate the time sequence, metamorphosis, localization, and some of the functional aspects of cellular elements during healing of an open wound by both light and electron microscopic observations.

MATERIALS AND METHODS

Thirty rats weighing approximately 180~200 grams were used for the experiments. Animals were wounded by excising 3.0×3.0 cm² skin, leaving subcutaneous alocular tissue, on the back following shaving of the hairs under ether anesthesia. The wounds were kept open without dressing. The tissue specimens were obtained from the wound at 6 and 18 hours, 1, 3, 5, 7, 10, 15, 21, and 28 days after the wounding.

For light microscopic examinations, a section across the middle of the original wound was fixed in 10% neutral formalin and processed for routine paraffin embedding methods. Microscopic sections were cut in 5 to 6μ thickness and stained by H-E, van Gieson, Masson's trichrome, alcian blue, PAS and Prussian blue methods for the observation of cellular and extracellular elements in wound healing.

For electron microscopic examination, small fragments, about 1 mm³ size, of tissue from the representative part of the wound were double fixed, first with 1.6% glutaraldehyde in phosphate buffer at pH 7.4 for 4 hours at 4°C, followed by post-fixation with 1% osmium tetroxide in phosphate buffer at pH 7.4 for 2 hours. They were dehydrated through graded ethanol and embedded in Epon. For the tissue orientation, 1μ thick sections from each block were prepared and stained with basic fuchsin. Ultrathin sections were cut with a glass knife and stained with uranyl acetate and lead citrate and were examined with Hitachi model HU 11-E Electron Microscope.

RESULTS

Light Microscopic Findings

For the convenience of description, the wound was divided into three layers, namely, the outmost layer of scab, underlying subscab which is mainly composed of exudates, and the innermost granulation tissue layer. Cellular elements of each layer at different intervals as well as extracellular elements are as follows.

Scab

The scab at 6 hours after the wounding consisted of thin but condensed layer of dense proteinaceous membrane at surface of the wound with aggregates of a few layers of polymorphonuclear leukocyte immediately underneath (Fig. 1, A). The nature of the proteinaceous membrane appears to be fibrin as characterized by strong positive reaction to PAS, deep red to trichrome, yellow to van Gieson and weakly positive to alcian blue. As the time passes this layer gradually became thicker by an increase of both the proteinaceous exudates and incorporation of large numbers of polymorphonuclear leukocytes(Fig. 1, B). A few macrophages were also admixed in this layer particularly in the lower level. The scab reached full thickness on the 1st day (Fig. 1, C) after the wounding and maintained the same thickness until the 7th day. From the 3rd day a tongue of regenerating surface epithelium underneath the scab at wound margin was noted. By the 10th to 15th day the epithelial regeneration was complete along with a separation of the scab layer.
The staining characteristics of the scab remained unchanged throughout the healing process, namely, strongly positive to PAS, deep red to trichrome, weakly positive or negative to alcian blue, and yellow to van Gieson.

**Subscab**

The subscab is the layer between scab and granulation tissue. At 6 hours after the wounding no definite outline of subscab can be delineated, but consists of markedly edematous loose connective tissue of original subcutaneous tissue. The cellular elements at this stage were mainly small numbers of polymorphonuclear leukocytes and a few mononuclear cells that were actively phagocytizing red blood cells and granular debris with swollen vesicular cytoplasm. Fibrocytes were those originally present, and became rounded up. The extracellular element was loose ground substance which gave a moderately positive reaction to PAS, strong positive reaction to alcian blue, and greenish blue staining to trichrome. Small amounts of reddish collagen fibrils were noted by van Gieson. But these collagen fibrils were apparently preexisting ones. There was also marked capillary engorgement without active proliferation.

This layer was relatively well delineated by the 18th hour after the wounding. At this stage the layer consisted of densely condensed proteinaceous extracellular elements which gave strong positive reaction to PAS, bright red to trichrome, yellow to van Gieson, and weakly positive to alcian blue. These characteristics were consistent with fibrin in nature. Within the fibrinous exudate, about equal numbers of polymorphonuclear leukocytes and macrophages, and scanty numbers of fibroblasts were noted. Mononuclear cells were markedly swollen with active phagocytosis of granular debris. The phagocytized particles gave the same staining characteristics as the surrounding fibrinous exudates. No stainable iron deposition was noted. On the 1st day after the wounding the subscab became thicker with the same staining characteristics as at 18 hours. The cells within this layer were then mostly mononuclear cells with active phagocytosis of the surrounding fibrinous material at the upper part and ground substance at the lower part which was continuous with newly formed underlying granulation tissue. There were scalloping hallows around the mononuclear cells in fibrinous exudate. Other cellular elements were small numbers of polymorphonuclear leukocytes, and mast cells (Fig. 2, A). On the 3rd day after the wounding, the layer became thickest and densely fibrinous. Cellular elements were mostly swollen mononuclear cells with active phagocytosis of fibrinous material and scalloping of surrounding fibrinous exudate. The exudate gave a very strong reaction to PAS, weak reaction to alcian blue, deep red to trichrome, and yellow to van Gieson. No demonstrable iron was present. Small numbers of polymorphonuclear leukocytes were also noted. No active fibrous ingrowth was noted (Fig. 2, B).

On the 5th day after the wounding the layer became thinner due to resorption from above and below as evidenced by marked scalloping and fibrous ingrowth. The cells were about equal in numbers of mononuclear cells and polymorphonuclear leukocytes. Mononuclear cells were less swollen but very active in phagocytosis. Numerous newly formed capillaries were invading into this layer from underlying granulation tissue, with prominent endothelial proliferation (Fig. 2, C). On the 7th day after the wounding this layer became very thin and the extracellular exudate became loose proteinaceous material.
giving still a strong reaction to PAS but a weak reaction to alcian blue, red to trichrome, yellow to van Gieson, and negative for iron. Cellular elements were mainly polymorphonuclear leukocytes, and mononuclear cells were less in number. There was extravasation of many red blood cells. Fibroblasts were actively proliferating into this layer. They were pleomorphic, some spindle and some stellate, and markedly disoriented. After the 10th day of wounding, this layer was completely replaced by newly formed granulation tissue.

Granulation tissue

Evidence of granulation tissue formation was first noted on the 1st day after wounding. Granulation tissue was formed beneath the subscab by activation of fibroblasts and endothelial cells. Besides the fibroblasts and endothelial cells, small numbers of mononuclear and mast cells were noted. The interstitium was loose and gave a weak reaction to PAS, and included small amounts of red collagen fibers which were apparently preexisting. On the 3rd day after the wounding subcutaneous fat cells appeared to transform into fibroblasts with perpendicularly orientation (Fig. 3, A). On the 5th day after wounding, the layer became very thick and consisted of actively proliferating fibroblasts and small numbers of mononuclear cells. The interstitium was loose and gave a weak reaction to PAS, yellow to van Gieson, reddish blue to trichrome, weak reaction to alcian blue and was negative for iron. The newly formed granulation tissue was growing into the subscab region accompanied by active capillary formation (Fig. 3, B and C). On the 7th day after the wounding, this layer became very thick with large numbers of pleomorphic fibroblasts, considerable numbers of polymorphonuclear leukocytes, and small numbers of mononuclear cells. Interstitium was loose, edematous, with numerous newly formed capillaries and extravasated red blood cells. The layer gave a very weak reaction to PAS, but moderately strong reaction to alcian blue, yellow to van Gieson, partly red and partly blue to trichrome due to early collagen formation. Newly formed capillaries were perpendicularly arranged (Fig. 3, D). After the 10th day of wounding, granulation tissue completely replaced the subscab. The cells were mostly hypertrophic spindle shaped fibroblasts with a parallel arrangement to the surface. Small numbers of mononuclear cells were also present. Interstitium was moderately dense and fibrous which gave a very weak positive reaction to PAS but moderately positive reaction to alcian blue, yellow to van Gieson, and dense blue to trichrome indicating a large amount of newly formed collagen fibers (Fig. 4, A). A small amount of stainable iron was noted at the lower part of the granulation tissue. On the 15th day after the wounding, capillaries were reduced in numbers, newly formed fine collagen fibers gave reddish staining to van Gieson (Fig. 4, B), and the amount of stainable iron was slightly increased. On the 28th day, collagen fibers began clumping (Fig. 4, C).

Epithelial regeneration

The first evidence of epithelial regeneration was noted on the 3rd day after the wounding as a spire of epithelial tongue from the margin of wound, penetrating underneath the scab and separating the scab and subscab (Fig. 5, A). As the regenerating epithelial front proceeded, it pushed off the overlying scab and completely covered the wound surface from the 7th to 10th day after the wounding (Fig. 5, B, C). The regenerated epithelial layer restored the whole layer of normal epidermis.
Electron Microscopic Findings

Electron microscopic observations were concentrated on the cellular elements.

Neutrophils

Neutrophils were predominant at 6, 18 and 24 hours after the wounding. They were enlarged about 10~12 μ at 6 hours and 15~24 μ at 24 hours. The nucleus was hypersegmented at about 18 hours (Fig. 6). The cytoplasm showed enlargement of specific granules, many small vesicles, and many phagocytized electron dense materials at 24 hours (Fig. 7). On the 3rd day, nucleus became very pyknotic and cytoplasm showed enlargement of granules. On the 10th day morphology returned to normal.

Mononuclear cells

From 6 hours to 18 hours after the wounding, mononuclear cells exhibited morphologic characteristics of blood monocytes except for an increase in the size and a very irregular cell surface. The nucleus was also slightly enlarged. The cytoplasm showed swollen mitochondria, loss of cristae and dilated golgi complex, many small vesicles, and enlargement of specific granules (Fig. 8).

After 18 hours, mononuclear cells showed morphologic characteristics of macrophages. The nucleus was enlarged containing a distinct nucleolus and the nuclear membrane was markedly indented. The cytoplasm showed a well developed rough endoplasmic reticulum, ribosomes and a few mitochondria (Fig. 9). From 24 hours to the 3rd day after the wounding, macrophages contained numerous and various kinds of phagocytized material. This debris was red blood cells, myeline figures, small vesicles, granular material, fibrin and other unidentified materials. Cell surface showed numerous villous cytoplasmic projections (Fig. 10).

Fibroblasts

The activated fibroblasts were first noted from the 3rd day after the wounding. At this stage they were rather immature as evidenced by the round or oval shape along with very scanty cytoplasm without fully developed organelles, but with a large nucleus (Fig. 11). On the 7th day the cytoplasm showed markedly dilated RER cisternae with densely attached ribosomes indicating increased functional activity (Fig. 12). On the 10th day the fibroblasts showed a fully mature appearance with well developed cytoplasmic organelles (Fig. 13).

Regenerating epithelium

After the 3rd day, the migration of the epithelial cells into homogenous, dense exudate took place surrounded by wide spaces (Fig. 14). Occasionally inflammatory cells were noted in distended intercellular spaces (Fig. 15).

On the 5th to 7th day after wounding, the intercellular space of regenerated epithelial cells became narrowed and many aggregates of free ribosomes appeared in the cytoplasm. Thereafter epidermal cells showed increments of tonofilaments and prominent mitochondria with irregularly dispersed cristae, and desmosomes were increased at the upper spinosum layer. After the 10th day the newly formed epidermal cells were indistinguishable from normal epidermal cells.

DISCUSSION

The process of wound healing has been described and reviewed extensively by nume-
rious investigators and much has been learned. The process of a simple and uncomplicated wound healing was observed in primary union, and the healing process of secondary union is similar in principle to primary union. The initial phase of primary wound healing is the filling of the incision gap with blood clots, followed by an acute inflammatory reaction, and the organization of blood clot by granulation tissue to complete the union. Concurrently repair of the surface epithelium takes place first by scab formation and then by epithelial regeneration. During these processes several cellular elements are involved.

The major cellular elements concerned with wound healing are polymorphonuclear neutrophilic leukocytes, mononuclear cells and fibroblasts. Time sequence of appearance of these cells, their function, and origin have been studied by various authors. Some of these were clarified, while others are still unknown.

Neutrophils are first to appear in response to tissue injury and wound healing, and they are the predominant cells during the first 2 days (Edwards and Dumphy 1958, Ross and Benditt 1961., Hirsch 1965). Normally neutrophils seldom appear outside of blood vessels in the skin and the origin is solely from the circulating blood. The well established function of the neutrophils is a front line defense force against invading agents, particularly microorganisms, and also lytic action of foreign materials or tissue debris by release of proteolytic enzymes stored in the granules. In addition to these proper function, neutrophils are thought to stimulate fibroblastic proliferation by releasing a sort of growth promoting substance “trephines” (Carrel 1921, 1922, 1924). Simpson and Ross (1972), and Page and Good (1958) studied the role of neutrophils in wound repair by comparing the effect of neutropenia on wound healing. They concluded that the only effect of neutropenia in wound healing was a decreased volume of fluid space and an increased numbers of extravasated red blood cells. But no effect on wound debridement or fibroblastic proliferation was noted. And the neutrophilic response in early stage of wound healing was not an essential antecedent to the infiltration of monocytes.

The neutrophilic response to open wound healing in our experiment showed that they are the first cells to appear at the 6th hour, and particularly they are concentrated on the surface of the wound immediately underneath a thin proteinaceous surface membrane, and later on mainly participated in the formation of scab. This phenomena appears to form a front line defense on the surface to protect against any agent that might invade from outside. Ultrastructurally, neutrophils in this early stage showed hypersegmentation with increase in size, and swelling of the granules. On the 1st day, there was phagocytic activities. On the 3rd day neutrophils underwent degenerative changes. Thus the major role of neutrophils in open wound healing appears to end after the 3rd day, and the role at the initial stage appears to form a front line barrier as the scab at surface and also participate in phagocytic activities to remove either foreign or exudated material to a certain extent.

The macrophages in wound healing is said to be from two sources. The first, a minor component, is the resident tissue macrophage which appears to be present in tissue at all times, and under a suitable stimuli is capable of entering the mitotic cycle and undergoing cell division (Ryan and Spector 1970, Volkmann 1971). The other, the major component, is directly recruited from hematogenous precursor cells, the monocytes, which themselves are derived from a rapidly dividing pool of
cells in the bone marrow (Volkman and Gowans 1965, van Furth and Cohn 1968).

The prime function of macrophages is phagocytosis of foreign particles of both invading agents and damaged tissue debris (Cline and Lehrer 1968). Some authors also consider that macrophage may transform into fibroblasts (Allgöwer and Hulliger 1960). Leibovich and Ross (1975) studied the role of the macrophage in wound repair by inducing monocytopenia by administration of hydrocortisone and antimacrophage serum. They reported that depletion of macrophages in the wound healing resulted in delaying of clearance of fibrin, neutrophils, erythrocytes and other wound debris, and delaying of fibrosis. They also stated that macrophages may stimulate fibroblastic proliferation by some unidentified manner.

In the present study, the mononuclear cells were the predominant cells from the 3rd day, and they were concentrated at the subscab which is mostly proteinaceous exudates containing large amount of fibrin. Ultrastructurally, the appearance of mononuclear cells at the early stage was that of blood monocytes as evidenced by lack of nucleolus and poorly developed RER. While mononuclear cells of later stage showed morphologic characteristics of macrophages as evidenced by larger cytoplasm with well developed RER, many vacuoles, and distinct nucleoli. Mononuclear cells of either monocyte or macrophage type, showed very active phagocytosis of all kinds of debris such as large amounts of fibrin, undigested red blood cells, and myelin like materials. Phagocytic activities continued until the subscab was resorbed and replaced by granulation tissue. Therefore, the major role of mononuclear cells in wound healing is apparently debridement of extravasated proteinaceous exudates and tissue debris at subscab level.

The fibrous replacement is the eventual outcome of wound repair and it is initiated by fibroblastic proliferation. The origin of fibroblasts during wound healing has long been controversial. The large mononuclear cells in blood, undifferentiated mesenchymal cells in the underlying fat and blood vessel adventitia were considered to be the origin of fibroblasts in wound healing. Recent studies on the parabiotic rats, however, indicated that fibroblasts in wound healing are not derived from circulating blood, but mostly derived from local tissue mesenchymal cells (Peacock 1970). In the present study, the origin of fibroblasts can not be determined. However, the fibroblasts appearing on the 3rd day were very immature, as evidenced by the large nucleus without development of cytoplasmic organelles. On the 5th to 7th day active protein synthesis was noted as evidenced by markedly dilated rough endoplasmic reticulum with densely attached ribosome. On the 10th day fibroblasts were well mature with abundant cytoplasmic organelles and newly formed fibers in the surrounding interstitium. Thus during the wound healing fibroblasts originated and differentiated from the immature mesenchymal cells finally to perform the prime function of synthesing fibrous proteins, namely, collagen and mucopolysaccharides.

REFERENCES


Legends of figures

Fig. 1. Process of scab formation. A: At 6 hours after the wounding showing thin proteinaceous surface layer and aggregates of neutrophils immediately underneath (× 430). B: Increased thickness of proteinaceous surface layer and underlying neutrophilic layers at 18 hours (× 430). C: Fully developed scab at 1st day, composed of a mixture of proteinaceous exudate and neutrophils (× 100).

Fig. 2. Macrophage activities at subscab. A: Loose proteinaceous exudates with small numbers of mononuclear cells on the 1st day after the wounding (× 430). B: Dense proteinaceous exudates with many swollen macrophages containing many phagocytized particles on the 3rd day. C: Ingrowth of fibrous granulation tissue at lower part of subscab with loose appearance of exudate due to resolution, and small numbers of residual swollen macrophages on the 5th day (× 430).

Fig. 3. Process of granulation tissue layer formation. A: Fibroblastic activation at subcutaneous fat tissue on the 3rd day (× 430). B: Marked resolution of proteinaceous subscab layer and active ingrowth of granulation tissue from underneath on the 5th day after the wounding (× 100). C: Higher magnification of Fig. B showing very pleomorphic fibroblasts growing into subscab. (× 430). D: Well developed granulation tissue composed mostly of plump fibroblasts on the 7th day (× 430).

Fig. 4. Process of collagenization: A: Junction between large old collagen bundles at the left and fine newly formed collagen fibers at right on the 10th day (× 430). B: Evidence of clumping of newly formed collagen on the 15th day (× 430). C: Well mature collagen with large bundle formation on the 20th day (× 430).

Fig. 5. Process of epithelial regeneration. A: Spire shaped regenerating epithelial front on the 3rd day after the wounding (× 430). B: Thin layer of regenerating epithelium in between scab and subscab on the 7th day (× 430). C: Complete reepithelialization on the 10th day (× 430).

Fig. 6-7. Neutrophilic polymorphonuclear cells showing hypersegmentation and granules with distinct nuclear membrane at the 19th hour (6), and phagocytized materials of amorphous, granular and lipid nature with apparent pseudopodia formation at the 24th hour (7). Mag. × 16,000 (6,7).

Fig. 8-11. Morphologic changes of mononuclear cells. Monocytic appearance of mononuclear cell at the 6 hours after the wounding, containing many small vesicles, cytoplasmic granules, and slightly swollen mitochondria (8). Mononuclear cell at the 18th hour showing morphologic characteristics of macrophages, namely, a larger amount of cytoplasm with abundant golgi complex, many phagocytized materials, such as granular debris, myelin figures, and red blood cells (9). Mononuclear cells at the 24th hour showing further characteristics of macrophages with moderately developed RER, villous cytoplasmic protrusions, and many phagocytized materials (10,11). Mag. × 16,000 (8), × 10,700 (9,10,11).

Fig. 11-13. Changes of fibroblasts. Immature appearance of fibroblasts with scanty cytoplasm at the 24th hour (11). A portion of elongated cytoplasm showing better developed RER and newly formed collagen fibers at extracellular area (12). Fibroblasted on the 5th day showing more mature appearance with abundant RER and many newly formed collagen fibers at interstitium (13). Mag. × 16,700 (11,12,13).

Fig. 14-15. Regenerating epithelial cells. A portion of regenerated epithelial cell on the 5th day showing many tonofilaments scattered throughout cytoplasm, a few mitochondria, some RER with many ribosomes, paucity of desmosome, and wide extracellular space (14). Regenerated epithelial cells on the 20th day showing a well developed appearance with many hemidesmosomes along the basement membrane, tonofilaments, free ribosomes and mitochondria (15). Mag. × 10,700 (14), × 22,500 (15).