HEALING AND REPAIR OF WOUNDS AND BONES

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Introduction

Wound healing and its physiology are principally of clinical interest. From the forensic point of view, the different phases of the healing process that occur chronologically but overlap, as described in 1867 by Cohnheim, provide information that is helpful when estimating wound age.

The timing of wounds is a classic problem in forensic histopathology and the literature on this topic is not easy to understand. This article discusses the physiological course of healing of human skin wounds and bone fractures from a forensic perspective. In addition, the associated morphologically detectable reactions of cellular and extracellular matrix components that are useful for age estimation are discussed.

Physiology of Skin Wound Healing

Wounds are a type of inflammation and their healing is characterized by an initial vascular phase, cellular reactions, and proliferative changes.

Early Vascular Phase

Immediately after severe tissue trauma, fibrin occurs in the damaged area as a result of the clotting process. However, fibrin can be induced up to 6 h after death, so fibrin detection does not unambiguously provide evidence of the vitality of a wound, even though some authors assume that vital fibrin reactions can be distinguished from postmortem ones by morphological criteria.

Every relevant tissue alteration is followed by an early vascular reaction leading to reduced tissue perfusion and enhanced vascular permeability. In this phase of decelerated blood flow, an interaction between hematogenous leukocytes and endothelial cells takes place, mediated by different cell adhesion molecules. In addition, proinflammatory cytokines such

as interleukins (IL-1β, IL-6, IL-8), growth factors (transforming growth factor TGF-β_1 or TGF-α, basic fibroblast growth factor), and tumor necrosis factor (TNF-α) act as peptide mediators and initiate a very early inflammatory response. The cascade of cytokines also regulates the induction of cell adhesion molecules and selectins (E-selectin, L-selectin, P-selectin) on activated endothelial cells and is therefore involved in the migration of leukocytes out of the vascular space. In this context, the interstitial cell adhesion molecule (ICAM-1) acts as a ligand of the leukocyte function-associated antigen (LFA-1), whereas the vascular cell adhesion molecule (VCAM-1) is a ligand of the very late activation antigen 4 (VLA-4). Different selectins are responsible for the attachment of the leukocytes at the vascular endothelium. After margination, the leukocytes actively permeate the vessel wall, attracted by several chemotactic agents such as degraded proteins, complement complexes, lymphokines, leukotrienes, thromboxanes, fibrin, and fibronectin.

Fibronectin is involved in leukocyte migration because of its chemotactic properties but it also provides a provisional matrix for migration of the leukocytes. It is synthesized by fibroblasts, endothelial cells, macrophages, and keratinocytes and shows a high affinity to collagen subtype III. Fibronectin enhances the attachment of fibroblasts and endothelial cells and seems to be involved in angiogenesis as well as in the contraction of the granulation tissue (Figure 1).

Cellular Reactions

On such a provisional matrix polymorphonuclear (PMN) cells infiltrate, followed by macrophages; chemotactic agents such as degraded proteins, complement, lymphokines, and thromboxanes are attracted to the damaged area. The PMN cells contribute to erythrocytes' lysis and phagocytosis of necrotic tissue by the release of proteinases and lysozyme. They also influence the permeability of the capillaries.

Although the migration of neutrophils and macrophages begins simultaneously, the reduced mobility of macrophages explains their later appearance in the wound area. However, neutrophils, which predominate in the early phase of the cellular reaction, quickly disappear from the lesion area, so that macrophages now dominate. These cells also release hydrolytic enzymes, complement and arachidonic acid derivates and are mainly responsible for the phagocytosis of necrotic tissue. Depending on the material incorporated, several subtypes can be identified after a few days. Lipophages show a typical foam-like cytoplasm while erythrophages are characterized by the presence of incorporated erythrocytes (Figure 2).

Inside the macrophages, the erythrocytes and their hemoglobin are degraded. This degradation process is induced by microsomal hemoxygenase and siderin results. This pigment, which is blue, can be detected in Prussian blue-stained sections and it is easily distinguished from crystallized bilirubin, so-called hematoidin. In contrast to hemosiderin, this pigment seems to be quickly reabsorbed and it is infrequently seen during wound healing.

Macrophages can be divided into subtypes with respect to their different phagocytic activities, but also according to the time-dependent expression of different immunohistochemically detectable antigens, for example, early (27 E 10), intermediate (RM 3/1), late (25 F 9), and chronic (G 16/1)-stage inflammation markers.

Although lymphocytes are mainly involved in chronic inflammation, they also play a part in healing wounds, although this is the subject of debate. Different definitions of a positive result and difficulties in distinguishing lymphocytes from neutrophils may be the reasons for reported differences. Therefore, only

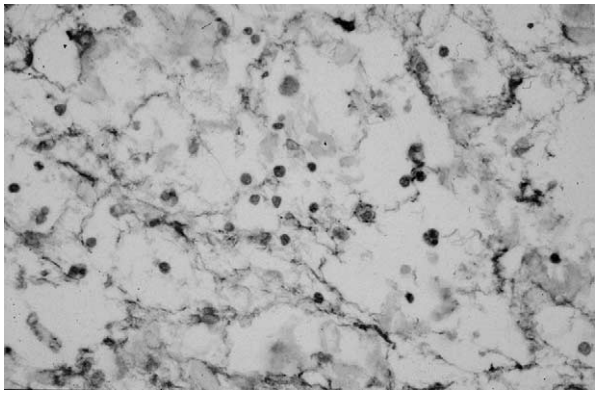

Figure 1 Human skin wound 12 h postinfliction. Typical network-like structures react positively for fibronectin and contain numerous neutrophil granulocytes (avidin-biotin complex (ABC), paraffin section, 380×).

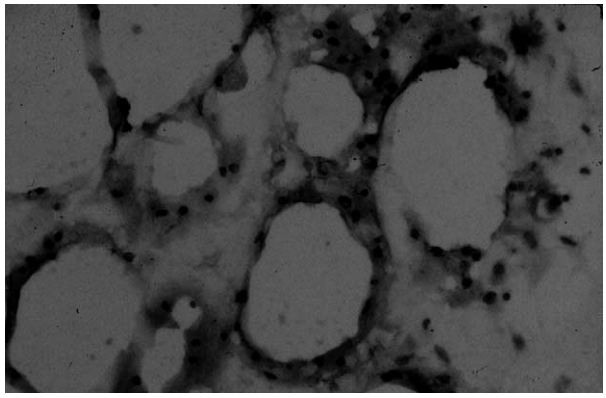

Figure 2 Human skin wound 5 days postinfliction: lipophages (frozen section, Sudan, 190×).

relevant lymphocytic infiltrates should be regarded as a reactive change.

In the area of the lesion, leukocytes, mainly PMN, and activated monocytes release proteins such as monokines and growth factors that, in combination with other factors such as fibrin degradation products, stimulate the proliferation and activation of fibroblasts.

Proliferative Changes

Proliferation can be detected by demonstration of the nuclear antigen Ki 67, which is expressed in the G_1-, G_2-, S-, and M-phases of the cell cycle and also occurs during physiological regeneration. Under pathological conditions, for example in wound healing, enhanced proliferative activity can be observed.

Cell proliferation in physiological or pathological conditions is closely associated with apoptosis. Apoptotic changes can be demonstrated by detection of the *p53* tumor suppressor gene, which arrests the cell in the G_1- or G_2-phase to enable DNA reparation. If the reparation processes are unsuccessful, *p53* initiates apoptosis and as a result DNA fragments can be found.

Fibroblasts proliferate, but also migrate into the wound area attracted by the chemotactic properties of collagen fragments, fibronectin, complement, and lymphokines. The activation of fibroblasts is mediated by fibrin or fibrin degradation products, respectively; but also by growth factors, thrombocytes, and macrophages. Activated fibroblasts are characterized by enhanced enzyme activities detectable by enzyme histochemical techniques. Under forensic conditions, a time-dependent increase in the activity of adenosine triphosphatase, nonspecific esterase, aminopeptidase, and acid as well as alkaline phosphatase has been investigated (Figure 3).

Additionally, activated fibroblasts synthesize several extracellular matrix components such as cell adhesion molecules like tenascin, which are mainly expressed during embryogenesis, and also in malignant tumors and during wound healing. Due to its typical structure combining domains of epidermal growth factor, fibronectin, and fibrin, tenascin seems to be involved in cell migration and in the regulation of cell adhesion as well as acting as a mitogenic stimulus.

Some fibroblasts differentiate into myofibroblasts which are contractile due to their actin content. They are involved in the stabilizing and contracting the granulation tissue by the expression of different basement membrane components such as laminin, heparan sulfate proteoglycan, and collagen type IV.

Fibroblasts also synthesize proteins to replace necrotic tissue, in particular different collagen subtypes. Collagens are proteins of the extracellular matrix with different molecular structures and are responsible for different biological functions. Collagen I is characterized by considerable mechanical properties while collagen III seems to be involved in wound contraction. Collagen V participates in the migration of capillary and endothelial cells during angiogenesis and mediates cell–substrate adhesion by binding on heparan sulfate proteoglycan. Collagen VI mediates the attachment of interstitial structures in the connective tissue and cell-binding activities (Figure 4).

At the same time as the damaged connective tissue is being repaired, keratinocytes are stimulated and activated in order to cover the epidermal defect by migration and proliferation. Migrating keratinocytes, which also contain actin-like contractile elements, are derived from intact superficial keratinocyte layers and from adjacent skin appendages. They also use a provisional matrix of fibrin and fibronectin, which is replaced by a basement membrane after

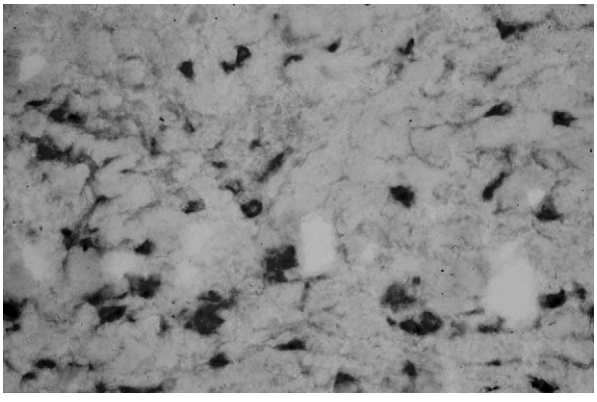

Figure 3 Human skin wound, 4 h postinfliction. Enhanced activity of nonspecific esterase in fibroblasts (frozen section, 380×).

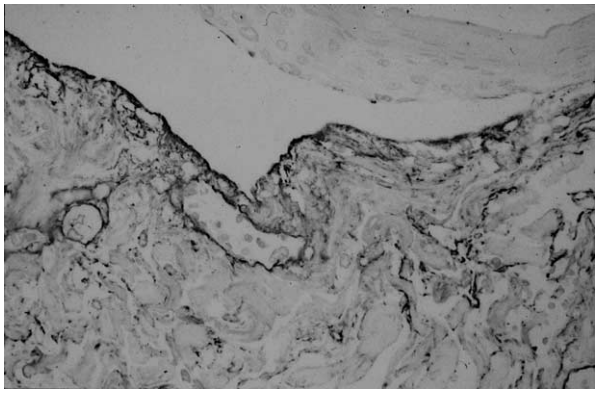

Figure 4 Human skin wound, 11 days postinfliction. Fibroblast-associated networks positively react for collagen type VI (ABC, paraffin section, 190×).

reepithelialization is complete. The basement membrane collagen types IV and VII are responsible for the mechanical stabilization of the newly built epidermis, whereas laminin and heparan sulfate proteoglycan are involved in cell–cell and cell–substrate interactions. The basement membrane components are mainly synthesized by the migrating keratinocytes, in contrast to the interstitial collagen subtypes, which are produced by fibroblasts (Figure 5).

Afterwards, the keratinocytes differentiate: the keratinocytes can be seen by the expression of keratin subtypes or other markers such as involucrin, filaggrin, and transglutaminase.

The initial cell-rich granulation tissue characterized by numerous fibroblasts and developing blood vessels is finally transformed into a permanent scar. During this process, enhanced apoptosis is responsible for cell reduction.

Bone Fracture Healing

Bone tissue is also continuously remodeled under physiological conditions, and the replacement of the organic and mineral bone matrix is rapidly enhanced during fracture healing. The remodeling process is mainly mediated by two cell types. Osteoclasts, derived from progenitor cells in the monocyte/macrophage system, are responsible for bone degradation by proteolysis of mineral and organic matrix. Osteoblasts are derived from progenitor cells normally located at the endost. These progenitor cells, mediated by cytokines, differentiate into osteoblasts or myelopoietic cells. Osteoblasts synthesize collagen type I but also temporarily subtypes III, V, and VI, as well as noncollagenous proteins during initial new bone formation. The balanced interaction between osteoclasts and osteoblasts in normal bone remodeling is significantly altered in fracture healing.

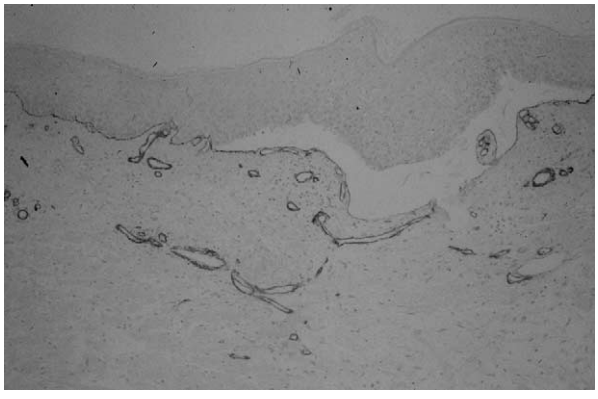

Figure 5 Human skin wound 3 weeks postinfliction. Rearrangement of the epidermal basement membrane, collagen type IV (ABC, paraffin section, 70×).

Physiological Course of Fracture Healing

Immediately after trauma, the fracture zone is filled by an extensive hematoma due to ruptured vessels of the periosteum and the opened bone marrow. This initial blood clot contains factors such as blood platelets. The thrombocytes release TGF-β, inducing the proliferation and differentiation of precursor cells into osteoblasts and chondrocytes. The bone morphogenic proteins (BMPs), also a part of the TGF-β-superfamily, additionally act on precursor cells. Furthermore, leukocytes (mainly PMN) are attracted to degrading necrotic tissue. Fibronectin degradation products mediate the migration of monocytes/macrophages and histiocytes as well as fibroblasts, leading to the formation of a granulation tissue that completely fills the fracture zone. Parallel to the increasingly condensing connective tissue ("fiber callus"), the fracture gap is usually widened. The bone margins that are irregular as a result of the fracture are smoothened by enhanced bone resorption, mediated by increased osteoclastic activity. This bone resorption is also essential for final stabilization of the fracture due to the removal of necrotic tissue and of sharp and irregular edges. The widening of the fracture zone results in an increased area of bone contact and contributes to the stability of the fracture zone since instable fractures undergo chondroid metaplasia. The resulting fibrocartilage callus is still biomechanically insufficient. The cartilage callus is secondarily transformed into an osseous one that is initially made up by woven bone but gradually replaced by maturing lamellar bone. Finally, this osseous callus proceeds to an increasingly maturing bone tissue completely bridging the fracture zone. Such a bridging callus can persist for a long time, particularly when the fracture ends are dislocated.

Principles of Forensic Wound-Age Estimation

As discussed, every phase of the healing process is characterized by the chronologic but overlapping appearance of several reactive changes and the time-dependent detection of these features is the basis of all wound-age estimations.

Even though the age of a certain lesion cannot be determined precisely due to various factors influencing the rapidity of the reparation process, such as individual age, malnutrition, malignant, or severe metabolic disorders, the postinfliction interval of a wound can be estimated by different criteria.

The earliest appearance of such a variable established in systematic investigations determines the minimum age of a lesion with a positive reaction. If a

variable regularly occurs in a specific time interval ("regular appearance"), i.e., such a reaction can be detected in every control specimen investigated, a postinfliction interval of less or more than that specific time interval is indicated. The latest appearance of a reaction can principally contribute to the estimation of advanced wound ages. However, this criterion is considerably influenced by the initial extent of the wound area and therefore it is of limited diagnostic value. From a practical point of view, it is almost exclusively the earliest appearance of reactive changes that is of forensic relevance since the diagnostic value of a positive finding considerably exceeds that of negative results, which may be absent in a section of a specimen but present in other parts of the lesion.

Several morphological features that are useful for wound age estimation can be detected by routine histological, enzyme histochemical, or immunohistochemical techniques. Routine histological staining procedures, for example, hematoxylin and eosin staining or the Prussian blue reaction, can easily be performed but a specific and unambiguous detection of several reactive changes is not always possible. The number of variables that can be demonstrated by enzyme histochemistry is also reduced and an irregular appearance of positive findings must be taken into consideration even though the advantage can be seen in the resistance to putrefaction. Immunohistochemistry is more sensitive to autolysis, depending on the antigen investigated, but allows specific detection of numerous parameters expressed during the healing process.

One of the most important conditions for a forensically useful age estimation of wounds is clear evidence of a positive reaction. Morphological features similar to postmortem changes are without diagnostic value.

Immunohistochemically detectable reactions must be evaluated critically with respect to nonspecific background staining or other artifacts. Typical network-like structures distant from the wound margins and outside the bleeding zone can be regarded as positive for the immunohistochemical detection of fibronectin, tenascin, or interstitial collagens, for example. Furthermore, the collagen reaction must be associated with fibroblasts because of the presence of destroyed connective tissue fibers in the damaged area. Negative as well as positive controls to prove the specificity of a reaction are necessary. Specimens showing relevant background staining cannot be evaluated.

The quality of the sections is of importance and it is only in optimally thin sections that a reliable evaluation is possible. In addition, a sufficient number of specimens per skin wound (in our opinion at least three specimens) must be investigated to confirm a negative finding. However, as mentioned above, conclusions based on positive findings are to be preferred. Therefore, Tables 1–4 give details of the earliest appearance of a useful variable.

Table 1 Variables of vitality of human skin wounds (postinfliction interval <30 min)

Variable	*Earliest occurrence*
Transforming growth factor-β1, interleukin-8, P-selectin	Minutes
Basic fibroblast growth factor, defensin 3	10 min
Fibronectin	10–20 min
Interleukin-1β, tumor necrosis factor-α	15 min
Interleukin-6	20 min
Neutrophil granulocytes	15–30 min

Table 2 Variables of shorter postinfliction intervals in human skin wounds (30 min up to 24 h)

Variable	*Earliest occurrence*
Vascular cell adhesion molecule-1, E-selectin, L-selectin	30 min
Interstitial cell adhesion molecule-1	50–60 min
Enhanced enzyme activity in fibroblasts	Hours
Macrophages	2–3 h
Macrophages > neutrophils	20 h

Table 3 Variables of advanced postinfliction intervals in human skin wounds (>24 h)

Variables	*Earliest appearance*
Fibroblast proliferation	1.5 days
Fibroblast apoptosis	1–2 days
Myofibroblast expression of laminin, heparan sulfate, proteoglycan	1.5 days
Tenascin	2 days
Migrating keratinocytes	2 days
Collagen III	2–3 days
Collagen V, VI	3 days
Lipophages, erythrophages	3 days
Siderophages, hemosiderin	3 days
Granulation tissue	3 days
Myofibroblast expression of collagen IV	4 days
Basement membrane fragments	4 days
Myofibroblast expression of α-actin	5 days
Complete reepithelialization (of surgical wounds)	5 days
Macrophage marker RM 3/1	7 days
Hematoidin, lymphocyte infiltrates	8 days
Complete basement membrane (in surgical wounds)	8 days
Macrophage marker 25 F 9	11 days
Macrophage marker G 16/1	12 days
Complete staining for keratin 5 (in surgical wounds)	13 days

Table 4 Course of normal fracture healing

Variable	*Estimated earliest occurrence*
Hematoma	Seconds
Neutrophil infiltration	12–48 h
Fibrohistiocytic proliferation	2–8 days
Collagen formation (fiber callus)	2–8 days
Chondroid metaplasia (cartilage callus)	1–4 weeks
Woven bone formation (osseous callus)	1–4 weeks
Lamellar bone maturation	5–9 weeks

Practical guidelines for the evaluation of immunohistochemical features have been established on the basis of extensive studies on human skin wounds, and have led to a considerable list of variables contributing to a forensic age estimation. The data reported for the time-dependent changes during fracture healing have not yet been confirmed in a similar way in forensic circumstances. Nevertheless, they can be regarded as a point of reference for the timing of bone fractures.

Further Reading

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