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REVIEW ARTICLE

Estimating wound age: looking into the future

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Abstract A critical review is made of the studies on wound healing used for forensic purposes, focusing on the problem of which characteristics indicate that a parameter could be used as evidence in court. A panel analysing the more important information obtained by each marker is given, and a perspective of what might be expected from future research is discussed.

Keywords Wound age · Research · Markers · Forensic evidence · Proof value

Introduction

Since the 1960s, new findings regarding inflammatory processes and the development of forensic histopathology have gradually resolved the uncertainty of the estimated age of wounds [1–10].

New histochemical, immunohistochemical and biochemical techniques have lead to studies focusing on wound healing, timing and differential diagnosis of injury inflicted ante- or post-mortem [11, 12]. As previously illustrated, no such parameter has yet been identified and we have to deal with substances and cells whose times of disappearance are too long, or that are present in the tissue under physiological conditions and, therefore, the positivity of the reaction must be based on percentage–quantitative data which are always debatable in court. Likewise, we must always consider that negative results may depend on individual factors or techniques applied. Hence, it is mandatory to examine the various parameters with different, but complementary, characteristics, so that the combination of results minimises the error margin in time calculation, in order that all confounding factors related to post-mortem phenomenon be avoided.

Forensic specialists and histopathologists first focused research on the quantification and/or qualification in tissues or fluids of the first cell movement; thereafter, they studied the substances involved in the cascade of events characterising the reaction of the organism to external or internal insults.

Wound healing

It is now well known that the inflammatory response and the tissue repair process, regardless of the damaging stimulus, depend upon a coordinated action over time in a series of molecules such as cytokines [interleukine-1 (IL-1), tumour necrosis factor (TNF), IL-6, IL-2, interferon-y (IFN-y), macrophage colony stimulating factor (M-CSF), transforming growth factor-β (TGF-β)], chemokines [α chemokines, chemokine β, lymphoaktine, fractalkine, IL-8, macrophage chemotactic protein-1 (MCP-1) and eotaxin], growth factors [heparin-binding epidermal growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor–scatter factor (HGF/SF)], members of extracellular matrix [fibronectin, collagen types I, III, IV and VII, elastin, fibrillin, proteoglycans, glycosaminoglycans (hyaluronic acid, heparan sulphate, dermatan sulphate, chondroitin sulphate), laminin], proteases, which play a role by binding through proteins such as integrins [lymphocyte function-associated antigen-1 (LFA-1), macrophage-1 antigen (MAC-1), very late antigen-4 (VLA-4)], phospholipids [platelet-activating factor (PAF)], or carbohydrates

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[carbohydrate sialyl Lewis x] to their receptors on the cells involved that in turn are activated [13–16].

Information emerging from general pathology studies regarding the repair process of skin wounds is reported in Table 1. If we compare timing of appearance and disappearance of inflammatory cells and of substances secreted during the inflammatory process emerging from general pathology with those obtained by forensic pathology researchers, it can be seen that there is a chronological discrepancy, with a shorter time trend described by the forensic pathologists. This is probably due to the different aims of these two branches of medicine. On the one hand, general pathology focuses on the mapping of action of all phenomena occurring in a sequencial fashion during the inflammatory response and their links, thus defining time with respect to the presence of these entities to be taken into consideration as effective for the action to be performed. Forensic pathology, on the other hand, focuses on the chronological mapping of gradual appearance and disappearance of these phenomena. Therefore, the mere appearance of cells or substances not normally present, that indicates a vital reaction of the body, although not sufficient to be effective, becomes of important forensic value in a court room setting.

A chronological study of wounds must always take into consideration, in the evaluation, local factors (size and type of lesion), and type of damaged tissue, as well as responsiveness of the individual, systemic factors that may influence the timing of wound healing (such as malnutrition) and clinical history (diabetes, immune deficiencies, vascular disease, collagen), medical treatment (antiblastic, glucocorticoids, radiation), and the subject's age that would appear to influence the course of the inflammatory response.

Indeed, experimental studies on mice have shown a decrease in the number and in the phagocytic capacity of macrophages and T lymphocytes with increased age of the animal, while neutrophil activity is similar both in the elderly and the young. The production of chemokines [macrophage inflammatory proteins (MIP-2, MIP-1α, MIP-1β and eotaxin)] also appears to be reduced with age in contrast to MCP-1 excretion which increases [18].

Table 1 Summary of major changes at microscopic level that occur after a skin lesion (data from [17])

Time since injury	Regulatory molecules	Substance produced or activated cells	Action
Few min	Histamin, serotonin, PAF, leukotrienes, thromboxane, prostaglandins PDGF, TGF-β, IL-1, TNF	Platelet activation. Coagulated blood with fibrin and fibronectin network that will serve as a "main road" to the inflammatory cells	Glue between the edges of the wound
Few h	PAF, PDGF, EGFs, TGF-β, HGF/SF, IL-1, TNF	Migration of survived keratinocytes. Basal layer of keratinocyte proliferation starts	Thin non-keratinized cell layer formation starts
24–48 h	PAF, factor XII, thrombin, EGFs, PDGF, TGF-β, HGF/SF, VEGFs, IL-1, TNF, IL-6, IL-10, TGF-ß, IL-1ra, Chemokines	Keratinocyte proliferation, neutrophil recruitment and macrophages that release proteases, lytic enzymes and molecules with regulatory action on inflammation	Completion not keratinized cell layer and underlying clot lysis
72 h	PGE, iNOS, PDGF, EGFs, TGF-β, HGF/SF, FGFs, VEGFs, IL-1, TNF, IL-6	Endothelial cell proliferation and migration that secrete proteases. Recruitment and proliferation of fibroblasts that deposit collagen type III at the edge of the wound	Additional space for clot lysis, neoangiogenesis and collagen deposit (granulation tissue)
1 week	PDGF, EGFs, TGF-β, HGF/SF, FGFs, VEGFs, IL-1	Peak proliferation of fibroblasts, which differentiate into myofibroblasts in wider wounds and deposit proteoglycans and collagen type I which replace type III and connect the edges of the wound. Early stage of regeneration of lymphatic vessels and of re-innervation	Increased thickness of keratinized epidermis, early formation of discontinuous basement membrane, complete replacement of the clot by granulation tissue. Top contraction of large wounds
2 weeks	FGFs, TNF, IL-6, IL-10, TGF-β, IL-1ra	Apoptosis of inflammatory cells and newly formed endothelial cells. Collagen I replaces collagen III up to a final ratio of 5:1	Full maturation of basement membrane and epidermis, regression of vascular network and progressive disappearance of leukocyte infiltration and fibroblasts
1 month		Acellular connective tissue with loss of appendages	Mature scar tissue
Several months		Stable relationship between collagen fibres	Increased resistance of the scar

Endothelial activation by chemical mediators

Endothelial cell activation is the earliest reaction of the inflammatory process and is triggered by chemical mediators which are involved in dermal–epidermal wound repair in the early hours after injury, contributing, therefore, to diagnosis of vitality. These mediators are substances, stored or newly synthesised, with short half-lives, such as histamine, serotonin, leukotrienes, prostaglandins (PG) [PGE1, PGE2, PGF2, prostacyclin (PG12)], thromboxane, PAF, inducible nitric oxide sinthase (iNOS), plasma proteases (Hageman factor, thrombin, and other inflammatory proteases).

Together with vasoactive amines and plasma proteases, a very important role is played by arachidonic acid metabolites. During the inflammatory process, a remodelling of phospholipids present on the cell membrane occurs as a result of the activation of phospholipases by mechanical stimuli or physical mediators such as C5a. During this process, arachidonic acid is released and further metabolised to form the lipoxygenase pathway that produces lipid with anti-inflammatory activity and leukotrienes which activate platelets and leukocytes, and cause vasoconstriction and bronchospasm.

Leukotriene-β4 is formed by activated phospholipids of endothelial cell membranes as well as of membranes of neutrophils, macrophages, lymphocytes and mast cells. LT-β 4, studied by high-performance liquid chromatography (HPLC) in injuries inflicted during life and post-mortem, is constantly present in vital lesions, while it is absent in those inflicted post-mortem. Therefore, it represents a useful parameter in the differential diagnosis in forensic pathology [19].

The phospholipid membrane separated from arachidonic acid has a powerful role as an inflammatory PAF, which induces platelet aggregation and activation, induces leukocyte migration and adherence, and activates the oxidative burst. It is expressed on endothelial cells, after inflammatory stimuli, such as histamine or cytokines. PAF is now being studied, particularly in anaphylaxis, as well as its metabolite, the precursor Lyso-PAF, and PAF acetyl-hydroxylase (PAF-AH) [20].

The cyclo-oxygenase (COX) pathway leads to the production of prostaglandins. Cyclo-oxygenase 1 (COX-1) is constitutionally expressed in many cell types and is, therefore, active in the very early stages of wound healing. Prostaglandins, important for wound healing, include PGE1, PGE2, PGD2, PGF2, PG12, and thromboxane (TXA2). PGE inhibits platelet aggregation, increases vascular permeability and induces vasodilation, while thromboxane induces vasoconstriction and promotes platelet aggregation. The high variability in the behaviour of prostaglandins, particularly PGF, however, does not make them suitable in the evaluation of wound timing [21].

An important role in this phase is also played by nitric oxide (NO). NO is produced by L-arginine through the endothelial cells NO synthase (eNOS), which is constitutively expressed, and through the iNOS of endothelial cells and macrophages activated by cytokines. NO induces the production of cyclic guanosine monophosphate (cGMP), which binds and stimulates several enzymes. It provokes vasodilation, reduces platelet adhesion and aggregation, in this way the pro-inflammatory signals become weaker by reducing leukocyte recruitment to the tissue.

Extracellular communication pathways through soluble mediators

Soluble mediators progressively enter into action after chemical mediators and persist over time, contributing to the diagnosis of injury timing. These are substances that act over short distances, binding the cells from which they are produced, or adjacent cells of different types, such as adhesion molecules [selectin P, selectin E, selectin L, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM)], growth factors [platelet-derived growth factor (PDGF), TGF-β, VEGFs, EGFs, FGFs, HGF/SF] and newly synthesised cytokines [IL-1, nuclear factor kB (NFkB), TNF, IL-6, IL-10, TGF-β, IL-1ra] and chemokines (chemokine α, β chemokine, lymphoaktine, fractalkine, IL-8 and MCP-1). In the assessment of timing their presence has to be searched in the region close to the injury.

Inflammatory cytokines represent an important family of soluble mediators. They are regulatory proteins that, together with growth factors and polypeptide hormones, have an active role in extracellular communication pathways. They are produced by several cell types (except IL-2 and IFN-γ, produced by lymphoid cells). Their production is transient and they act at a short distance, except IL-6 which acts on the liver. Under physiological conditions, they are present in small quantities in blood circulation, except TGF-ß and M-CSF which are present in large quantities. They are often pleiotropic because they act on a wide variety of cells and tissues and, although structurally different, they often have superimposed action, e.g. IL-1 and TNF.

The primary inflammatory cytokines are IL-1 and TNF, which have many features in common, as well as IL-6. They are rapidly activated, act in small concentrations (nano and picomolar) and have a half-life of about 3 min. The forensic interest lies in the characteristic of the positivity shown by inflammatory cells. In particular, in the early hours, positivity dominates on neutrophils, which are gradually replaced by positive macrophages and then by fibroblasts. Therefore, the observations on the behaviour of each cell type will provide information regarding the age of the lesion.

To obtain this information, the methodology used must be chosen carefully. Studies using methods of quantitative analysis, such as enzyme-linked immunosorbent assay (ELISA), show surprisingly different results compared to

immunohistochemistry (IHC), as the quantification of cytokines does not take into account the cell type producer. Considerable differences are also observed based on the type of injury inflicted. ELISA shows higher increases of IL-1β, IL-6 and TNF found in deadly weapon wounds even after less than 5 min, while in surgical wounds an increase in IL-1β is seen within <30 min and of TNF-α in 1–2 h. This difference could be related to the stress reaction of the body against an injury that is not controlled by anaesthesia [22].

Studies with reverse transcription polymerase chain reaction (RT-PCR) and in situ hybridization (ISH) confirm the increasing values of all cytokines immediately after trauma with a peak between 48 and 72 h for IL-1α and β and TNF-α, whereas IL-6 shows a peak at 6 h [23].

A correct evaluation of the results needs to include comparison with intact skin taken from the same individual, as the inter-individual variation of activities have proved relevant, and, therefore, assessment of individual base values is necessary. IL-6 is a cytokine not present on healthy skin and, therefore, its variation is the most reliable.

All cells, especially monocytes and macrophages, possess trans-membrane receptors for IL-1 which have the intra-cytoplasmic task to activate, through a cascade of kinases, the pro-inflammatory transcription factor, NFkB complex, located upstream in several inflammatory genes which, in turn, are activated [24] (Table 2).

IL-1α is positive on keratinocytes and sweat glands in healthy human skin and thus does not provide important information regarding wound timing. Experimental studies on mice, however, show a peak of IL-1α at 6 h and a rebound of activity within 72 h on fibroblasts [25].

More interesting results have been provided by neutrophils which never stain positive for IL-1α in wounds aged <30 min, but are well-represented in lesions from 4 h to 1 day, with a peak at 7 h. As the time since lesion affliction increases, macrophages and fibroblasts replace neutrophils, with a gradual decrease in the ratio of infiltrating positive/negative cells from day 1.5. A finding of 30% positive inflammatory cells would indicate a lesion of <1 day. No differences related to sex, age or type of injury are found [26].

IL-1β is absent or barely present on the keratinocytes of healthy skin, while cells of the dermis, capillaries and excretory ducts of sweat glands may be positive. At IHC, an initial positive reaction is observed after 15–20 min, with a peak (>50%) at 30–60 min on keratinocytes of the basal and spinous layer, followed by those of the granulose layer, up to 90 min after the injury [27]. Experimental studies on mice have shown a rapid increase of IL-1β with a peak at 3 h and a rebound in activity after 72 h on fibroblasts [25].

The biological action of TNF is similar to that of IL-1 except for the activation of apoptosis and inhibition of haematopoietic precursors, which are activated by IL-1.

TNF exists in two isoforms: TNF-α and TNF-ß or lymphotoxin. TNF-ß is produced by lymphoid cells, and TNF-α primarily by mononuclear phagocytes. The interaction with the receptor type I or p55 activates a programme of apoptotic death by activating the caspase cascade of proteolytic enzymes. The interaction with the receptor II or p75 inhibits apoptosis and activates NFkB.

Studies on the expression of TNF-α on keratinocytes show little positivity on healthy skin with frequent negativity. An initial positivity occurs 15 min after injury with a peak at 60–90 min [22–27]. A significantly increased expression of TNF-α is seen on skin mast cells in lesions of 5 min, with a peak at 1 h [28]. Experimental studies on mice show a peak at 3 h and a rebound of activity in 72 h on fibroblasts [25].

IL-6 is a secondary mediator of IL-1 and TNF, produced mainly by monocytes and macrophages, which activates gene transcription for megakaryocytic differentiation and for the production of B and T lymphocytes. On vascular endothelium, IL-6 amplifies the expression of adhesion molecules and chemokines active on monocytes but not on neutrophils, hence allowing the passage from acute to chronic inflammation.

IL-6 stains positive in few keratinocytes on healthy skin (<10%), while it is significantly positive (10–25%) at 20 min after the injury, with a peak at 60–90 min but still persisting at 5 h [27]. Experimental studies on mice show a peak at 12 h and a rebound of activity within 72 h on fibroblasts [26].

IL-10, TGF-α, TGF-ß and IL-1ra are anti-inflammatory cytokines produced by monocytes and macrophages and by the same stimuli that activate IL-1 and TNF. Glucocorticoid hormones, IL-13 and IL-4 increase the production of anti-inflammatory cytokines.

Table 2 Secondary mediators induced by NFkB

- Inflammatory cytokines (IL-1, IL-6) with amplification of the response
- Inflammatory chemokines (IL-8, MCP-1, etc.)
- Endothelial adhesion molecules (E selectin, ICAM-1, ICAM-2, VCAM)
- Enzymes that produce effector molecules (iNOS, COX-2)
- Molecules that activate the immune response (CD80) and haematopoietic growth factors (GM-CSF and M-CSF)
- Lipid mediators (prostaglandins and NO) which in turn amplify leukocyte recruitment which leads to the activation of innate immunity that induces type I immunity (especially by IL-12), which produces IFN-γ and type II immunity which produces IL-4 and IL-13

TGF-α is weakly expressed in normal skin and shows a marked increase in epidermal reactivity after a wound age of approximately 10 min with a peak at 30–60 min. TGF-ß1 is constitutionally expressed only in connective tissue, and shows an increase after several minutes, presumably due to an infiltration with TGF-ß-rich thrombocytes of the traumatised skin and the epidermal layers, with a peak at 30–60 min. Both factors are detectable in elevated levels also in older wounds with an age of days to weeks (network in granulation tissue) [29].

Experimental studies on mice have shown increased levels of IL-10 mRNA measured by RT-PCR already at 15 min with a peak at 60 min. The levels found between 30 and 180 min were considered significant. The method proved reliable for vitality also in injuries on tissues affected by post-death phenomena [30].

A recent study using a multiplex bead-based immunoassay attempted to build a grid of simultaneously cross-evaluated results obtained with IL-2, IL-4, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, and TNF-α in injured human skin. There was an initial neutrophil and macrophage infiltration at 2 h, which increased at 33–49 h. T and B lymphocytes infiltrated simultaneously at 71 h. Fibroblasts follow from 246 h. IL-10, GM-CSF, IFN-γ, and TNF-α were increased in the first stage, IL-6 in the intermediate stage and IL-2, IL-4 and IL-8 in the medium-late stage [31].

A superfamily of 50 proteins with chemotactic activity defined chemokines can be found among cytokines. The α-chemokines are active on polymorphonuclear cells and T and B lymphocytes. Instead, β-chemokines are active on monocytes, lymphocytes, N cells, eosinophils and basophils, while lymphoaktine and fractalkine on T lymphocytes and NK cells, IL-8 on neutrophils, MCP-1 on monocytes and eotaxin on neutrophils.

In the presence of IL-1 and TNF, chemokines can trigger the production of reactive oxygen intermediates and amplify the inflammatory response by activating the transcription of genes for chemokines and proteases.

Kondo et al. [32] studied human skin lesions focusing on IL-8, MCP-1 and MIP-1α, and they found that neutrophils showed positivity for all three markers in injuries of 4–12 h. Later in time, macrophages and fibroblasts gradually replaced neutrophils, at a significant positive/negative cell ratio between 1 and 4 days. The most significant relationship to injuries was reported between 7 and 21 days compared to those between 0 and 12 h. In brief, a 50% rate of positive cells for IL-8, 30% for MCP-1 and 40% for MIP-1α indicates a lesion at least 1 day old.

Cell adhesion molecules

Cell adhesion molecules are proteins expressed by endothelial cells which allow leukocyte recruitment during inflammatory response. They are expressed in particularly high concentrations from post-capillary venules, where the junctions between the cells are rather loose and, therefore, the passage of leukocytes is much easier. Their activation depends on the type of stimulus they receive (blunt trauma, bruises, bacterial infection, allergic reactions, etc) [33, 34].

P-selectin (CD62P) is a glycoprotein naturally present in Weibel and Palade corpuscles of endothelial cells and in secretory granules of platelets. When endothelial cells or platelets are stimulated by histamine or thrombin, there is a rapid melting (1–2 min) of the bodies of Weibel–Palade with the plasma membrane that causes the translocation of P-selectin on the surface within a few minutes (peak 10–15 min) which then decreases over an hour to physiological levels within 2–4 h. Once it reaches the cell surface, it binds the integrins (Lex) of neutrophils and monocytes. It is not induced by cytokines such as IL-1 and TNF and is, therefore, essential for the early binding of leukocytes, independently of cytokines, in the sites of wound healing. This characteristic makes it a good parameter for assessing vitality of injuries inflicted in liminae vitae.

Experimental studies, at very brief interval of time, on human skin detected an increased positivity of endothelial cells already at 1 min with a more evident increase at 10–15 min [35]. Other studies recorded significant positivity between 3 min and 7 h [36]. Evaluation of positivity of the reaction needs to be compared to the normal skin of the same individual as there is a large inter-individual variation of P-selectin expression, and basic levels may also express the reactivity of the individual to the insults. In a recent study by the author, high constitutional levels of P-selectin may be accompanied by a more precocious activation of E selectin (data not shown).

E selectin (CD62E) is a glycoprotein newly synthesised by endothelial cells of post-capillary venules and of venules of lymph nodes only after inflammatory stimuli such as cytokines IL-1, TNF or lipopolysaccharide. Its role is to bind the integrins in neutrophils, monocytes and T cells. Its expression is dependent upon protein synthesis and is the first molecule to be induced by TNF within 1–2 h, reaching a peak after 4–6 h of stimulation and then declining until it can no longer be measured after 8–10 h. Finally, it can be traced for a period not exceeding 24 h after the inflammatory irritation.

In a clinical study, Van der Laan et al. [33] showed that a surgical incision produces non-specific acute inflammation characterised by the presence of E selectin positive venules in which granulocytes infiltrate the interstitium already at 30 min. Furthermore, Takeuchi et al. [37] showed that 1 h after a small skin trauma the level of m-RNA for E selectin is up-regulated and persists at 4 h, with increased expression of E selectin in the vessels of the dermis compared to healthy skin by IHC. A simple stretching of keratinocytes might

release IL-1α, which stimulates the synthesis and expression of selectin E. Forensic pathology studies have also shown the activation of selectin E from 1 h up to 17 days with a significant decrease after 2 h [36]. Considering that selectin E is absent in healthy skin, it is an important marker of lesion vitality.

Another cell adhesion molecule that should not be overlooked is ICAM-1 (CD54). This is an immunoglobulin, with adhesive properties, present constitutionally on endothelial cells to ensure vasal tone and leukopedesis, the concentration of which increases after stimulation with IL-1 and TNF. Its role is to bind integrins (LFA-1 and MAC-1) in neutrophils, monocytes and lymphocytes. ICAM-1-deficient mice present an inhibition of the inflammatory process with reduced infiltration of neutrophils and macrophages, no migration of keratinocytes and no granulation tissue formation [38]. ICAM-1 may be present in healthy skin even in low concentrations on endothelial cells and keratinocytes, and on activated leukocytes. A clearly increased positivity, on all these cells, is observed in lesions from 1.5 h to 3.5 days, but it is not suitable as a single parameter [39].

The VCAM (CD106) is also an immunoglobulin with adhesion functions and, after stimulation with IL-1 and TNF, binds the integrin VLA-4 or α4β1 on monocytes and lymphocytes. Not normally present on healthy skin [33], it can sometimes appear with mild intensity on vessels of healthy skin, and increases after a lesion with a clear reaction for 3 h after injury and with a peak between the fourth and sixth hour, thereafter decreasing slowly up to 3.5 days [40].

L selectin (CD62L) or leukocyte adhesion molecule (LAM) is a protein expressed on circulating leukocytes, especially non-activated T lymphocytes. Its ligand on endothelial cells is a proteoglycan, glycosylation-dependent cell adhesion molecule-1 GlyCAM-1, in particular, on high endothelial venules of peripheral lymph nodes, in which it promotes the recirculation of lymphocytes. In sites of wound healing, L selectin expressed on neutrophils favours the binding of these cells to endothelial cells activated by cytokines (IL-1, TNF and IFN). It allows the passage of leukocytes through the endothelial wall. It is always found on activated leukocytes of healthy skin and lesions [36], thus making it unsuitable for lesion timing.

Finally, growth factors have been examined and positivity to bFGF was detected in the nuclei of keratinocytes and fibroblasts already at 30 min–1 h and up to 24–144 h. VEGF was positive in the cytoplasm of epidermal cells at 24–144 h. Both growth factors are present after day 1 [41], while over 50% of macrophages and fibroblasts expressed VEGF in the cytoplasm after 7 days [42].

Recently, a molecule involved in the earliest stages of wound healing, produced by damaged vessels, called vascular adhesion protein-1 enzyme, was identified. It has an oxidative activity and appears to be activated by inflammatory vascular lesions, and stimulates the up-regulation of time-dependent selectin transcription [43]. Research needs to be carried out to establish its effective use in wound timing.

Techniques and methods

Early studies on wound vitality have used basic histological techniques and focused on the assessment of cell movement. The first observations were made by Walcher [44] and later by the Finnish school of Raekallio (reported in [12]). The German school was quite active during the 1970s, 1980s and 1990s, in this field, and the results obtained were outlined on several occasions by Betz [6].

Data obtained by Raekallio in the 60 s (later reported in [2]) reduced the time from the injury to 1 h by studying enzymes produced by cells, using histochemical methods, while microspectrophotometry used later better clarified enzyme appearance timing [45–47].

Biochemical methods have been adapted to evaluate concentration levels of vasoactive amines during wound healing. Early studies on histamine and serotonin date back to the school of Berg (reported in [1, 48–50]). Later, histamine was studied with more sophisticated methods such as microfluorimetry [51], HPLC [52], spectrophotometry, and spectrofluorimetry [53] with contradictory results. Atomic absorption spectrometry was used in the investigation of ions such as iron, zinc, magnesium, sodium, potassium, and copper demonstrating the usefulness of iron as a parameter of vitality and of the ratio K/Na [54–56].

Cellular infiltration

Using IHC, the field of wound timing investigations has expanded dramatically. With IHC it has been possible to pinpoint, more precisely than with other techniques, the temporal movement and cell proliferation in wound healing.

In addition to the identification of different cell types in the context of inflammatory reaction, as synthesised by Betz [7], IHC has made it possible to study the chemical mediators (histamine) that are secreted early or produced, and the profound changes that occur to the extracellular matrix components in the course of wound healing (protein collagen types I, III, V and VI, laminin, proteoglycan heparansulphate, vimentin, desmin, etc.) [57]. It has also made specific localization of tissutal substances indicative of the stage of response (fibrin, fibrinogen, fibronectin, tenascin, complement, different types of collagen, etc.) possible [58–64] and the phases of activation of individual cells (positivity of myofibroblasts for laminin, heparan sulphate proteoglycan, collagen type IV, α—smooth muscle

actin, or macrophages in various stages of activation positive for myeloid-related proteins (MRP-8, MRP-14), 27E10, integral membrane protein (RM3/1), 25F9, etc.) [65]. The latter, however, has not provided additional information for forensic, compared to basic, histology [59].

Nuclei strongly positive for ubiquitin (Ub) in neutrophils, but not in macrophages and fibroblasts, were shown between 4 h and 1 day. A percentage of positivity for Ub >10% indicates a lesion of at least 1 day, while >30% 7–14 days [66].

A number of studies using various antibodies have been performed in attempt to define wound timing. For example, fibrocytes, bone marrow-derived mesenchymal progenitors that coexpress hematopoietic cell antigens and fibroblast products, were recently studied by double-colour immunofluorescence analysis using anti-CD45 and anti-collagen type I antibodies. Results revealed the absence of fibrocytes in human skin wounds until day 4, with a peak at days 9–14 [67].

Furthermore, the migration of melanocytes, within scar tissue, was studied using S100 polyclonal antibody showing a maximum at 1.8 years in scars (mean 1.85–1.94), thereafter gradually decreasing to basal within about 10 years [68].

p53 protein stops the cell cycle when DNA is damaged and triggers cell apoptosis. An initial expression of p53 in fibroblasts is shown at day 3 after injury, while high expression is observed on day 8 and stabilisation, at high levels, between 3 and 11 weeks. Positive reaction persists also in the wounds of more advanced ages [69, 70] thus providing helpful data in establishing when the wound occurred.

One hundred fifty kilodalton oxygen-regulated protein (ORP 150) is a novel stress protein that is located in the endoplasmic reticulum and contributes to cell survival when this organelle is under stress. Studies on human skin wounds demonstrate a macrophage and fibroblast ORP150-positive ratio >50% in wounds aged 7–14 days [71].

Several studies have focused on the increasing numbers of mast cells with advancement of wound timing. Using anti-triptase and anti-chimase antibodies an increase in mast cells is observed in the period immediately following the lesion, with a peak at 1–3 h later decreasing within 6 h [72, 73]. However, factors such as the post-mortem release of proteins from human mast cells needs to be taken into account when interpreting the data [6].

These problems can be avoided by investigating the stem cell factor (SCF) and the Kit receptor molecules necessary for the survival, growth, migration, and activation of mast cells, which offer the possibility to define the vital activation of mast cells, apart from enzyme release. The results revealed a rapid increase in cells positive for SCF in the dermis of lesions by day 1 and thereafter the decrease to baseline. The Kit receptor grows more gradually, with a peak on day 14 [74].

Recently, the time-dependent loss of mast cell enzyme activity at the margin of wounds was evaluated by IHC comparing the number of triptase-reactive mast cells, which do not lose all their enzymatic activity during degranulation, to naphtol AS-D chloroacetate esterase (NASDCAE)-positive mast cells, which lose all enzyme activity after activation. Results show that in injuries of <60 min, the number of NASDCAE-positive mast cells, near the margin is significantly lower than that of triptase reactive mast cells, thus correlating inversely with the distance from wound edges [75].

By combining different methods and techniques it is possible to obtain further new information regarding timing. Experimental studies have focused on the proliferation of basal cells of the epidermis through the release of the nucleolus organiser regions with silver impregnation techniques (AgNORs) and IHC techniques with Ab anti-bromodeoxyuridine (BrdU). The results showed good assessment of the lesion between 10 and 96 h, with a plateau between 36 and 70 h [76].

Single cell stage activation

Molecular biology is now able to provide new opportunities to obtain data and carry out investigations on wound timing by allowing definition of the stage of activation of the cell, and which mediator is to be synthesised. This information could be used in court to prove both the vitality of the reaction and the inflammatory phase.

Use of this technique has already given some interesting results. The quantification of RNA synthesis by an in vitro technique with 3H-cytidine, for instance, confirmed a significant incorporation only in viable keratinocytes of the basal layer in lesions of 10–24 h, while in post-mortem injuries, there was no synthesis of RNA [77, 78]. Cells undergoing DNA synthesis (S phase) are able to incorporate BrdU. A peak increase of DNA synthesis, between 32 and 60 h after injury, was seen in skin taken in vivo and post-mortem from rats. Post-mortem DNA synthesis, in vital injuries, implies that the mechanism of induction will continue even after death. This element can be used as an index of vitality, but not for timing, because of the uncertain correlation with the interval following injury [79].

In situ end labelling (ISEL) and terminal deoxynucleotidyl transferase Biotin-dUTP nick end labelling (TUNEL) techniques offered the possibility to study the phenomenon of apoptosis, which is necessary for completion of the inflammatory process and tissue regeneration, in order to verify its application for forensic purposes. An experimental study in rats showed a significant increase in the number of apoptotic cells in skin affected by scratches compared to that in normal skin, with a significant involvement of keratinocytes in

lesions of 2–6 days old and cells of the dermis from day 3–8. This indicates a period of quiescence before the beginning of apoptosis [80]. Studies on human skin have detected apoptosis of neutrophils on day 2 after injury [81], while apoptosis of fibroblasts became significant after 3 weeks [82] and keratinocytes initiate apoptosis after 120 min in 50% of cases [83]. Results of this study are in contrast with the fact that the physiological apoptosis of keratinocytes, typical of all highly proliferative tissues, allows the cell turnover of the epidermis.

The expression of proto-oncogenes c-fos and c-jun, which are very early response genes, is constitutionally found also in the normal skin in nuclei of epidermal cells of the basal layer. After injury, they first appear in the nuclei of neutrophils, and macrophages, which infiltrate the basal layer. Thereafter, they appear in the nuclei of monocytes and fibroblasts, indicating the time of the lesion with a peak after the first day [84].

Timing of injury and limitations of forensic evidence

The timing of injury has to be sustained by evidence that can be presented as proof in court. For this purpose, it is essential to take into account the accumulated experience which has ensured greater value of evidence in the trial, in which the parameters used (cell type or substance) are absent in physiological conditions, appear regular in time and disappear after a given time. Knowledge concerning the regularity of its occurrence, presence and disappearance (time limits) provides reliable information, particularly if absent, which indicates timing of the lesion, less than or greater than the known time limit for that specific parameter.

The contradictory results obtained, in some cases, in the various studies suggest that intrinsic and extrinsic factors, methodology used and the time elapsed from death all influence the results. Each technique and each parameter need a different approach in the assessment of results. In applying IHC, for instance, negative controls, healthy skin and internal controls, of the same individual, should always be studied together with the wounded skin. The latter provides indispensable information regarding the physiological presence of the parameter. Positivity should be evaluated considering the co-presence of the cells linked to them. False positives can be seen on the margins of lesions thus suggesting post-mortal artefacts or contamination with serum components, e.g. as occurs with proteinase inhibitors such as the α_1-anti-chymotrypsin and $\alpha 2$-macroglobulin, or with fibronectin, but does not occur with lysozyme [85]. In fact, studies on these substances did not give entirely reliable results [62, 86].

When studying cell movement, the potential post-mortem passage of granulocytes, macrophages and lymphocytes from the blood to the tissue has to be taken into consideration when assessing the data. Thus consequently, only a significant presence, in the form of infiltration of individual cell types, may be indicative of vitality. Furthermore, in some cases, cell movement is individual-dependent and also lesion-size dependent, as in the migration and proliferation of keratinocytes, therefore, the values obtained should be considered with caution.

When studying markers of enzyme movement, it should not be forgotten that they suffer from post-mortem phenomena. Furthermore, the considerable variability in results makes enzymes useful only when positive and when it can be assumed that they indicate an interval of several hours after the injury [6].

Some markers can be influenced by pre-existing pathological conditions, or can be produced also post-mortem. Fibrin, in fact, may not be present in death related to bleeding disorders, whereas it can be found in post-mortem wounds. On the other hand, post-mortem injuries can induce collagen production due to chemical and physical changes in the fibres, thus making this element unreliable for forensics. In cases like these, it is useful to search for the possible presence of an intermediate metabolite. For example, as far as concerns fibrin, the presence of the D-dimer can be considered a sign of vitality in injuries due to a cut, while in the case of bruises or abrasions, results did not appear to be satisfactory [87].

Sometimes, it is necessary to distinguish between vital and post-mortem injury and thus a marker is needed that is not formed in the post-mortem period or in post-mortem injuries. Cathepsin D followed by cathepsin A are useful in distinguishing vital from post-mortem injuries in animals as well as on human skin lesions only a few minutes old [55].

When evaluating the various parameters that have been studied to assess the vitality or the timing of a skin lesion, additional factors need to be taken into consideration.

First of all, the inflammatory response varies as far as concerns the time interval of cell movement and molecule expression, but not in the sequence of events, thus depending on the type of lesion, its extent and often on the individual reactivity. Another factor concerns the increase in age of the subject, which appears to reduce the activity of macrophages and T lymphocytes. Unfortunately, due to the marked differences in the time of appearance, peak and decrease of positivities, it is impossible to use these parameters as evidence in court. A final consideration is that the absence of one or more parameters often provides more information than its/their eventual presence, thus offering the possibility to establish beyond reasonable doubt that the lesion had been provoked much earlier or much later with respect to the moment in time in which the parameter/s were certainly present. When evaluating a new parameter, all four of these aspects need to be taken into consideration.

Table 3 Most reliable information on timing in wound healing reaction of markers of cell movement (*), enzyme movement (°), reactions in the extracellular matrix (^) and endothelial adhesion molecules (#)

Wound age estimated in minutes

- *TNF-α* positive mast cells at the site of trauma*: 5′, peak 1 h
- Haemorrhagic infiltration of neutrophils out of the area*: >15′
- Keratinocytes *IL-1β* positive, on basal and spinous layer first, then on granulous*: >15′–20′, peak (>50%) to 30′–60′ up to 90′
- Keratinocytes *TNF-α* positive*: >15′, peak 60′–90′
- Keratinocytes *IL-6* positive*: 20′, peak 60′–90′, still present at 5 h
- *Acid 5-hydroxy-indoloacetic*°: HPLC doubling baseline at 10′
- *Histamine*°: excessive variability technique-dependent. With HPLC: > 15′–30 ′, with IHC: > 100% at 1 h. Not reliable marker
- *Serotonin*°: detectable with enzyme histochemistry <5′, decreases up to 15′; with HPLC: peak to 10′–30′ up to 24 h
- *Tenascin* and *fibronectin* in a grid around neutrophils and fibroblasts^: lesion vital
- *Fibrinogen*^: by 1′, but unreliable
- *Fibrin*^: > few minutes, but also in post-mortem wounds
- *Fibronectin*^: > 20′, a positive trend in the post-mortem injuries is always present
- *Selectin P* #: 1′–3 ′, peak 10′–15′, physiological levels at 4–7 h
- *Selectin E* #: initial 5′–10 ′, evident at 30′–1 h, peak at 4–6 h, decrease at 6–10 h, absent >24 h

Wound age estimated in hours

- Low NAS-DCAE-positive mast cells/tryptase reactive mast cells*: <1 h
- Mast cells: *anti-triptase* and *anti-chymase* positive*: peak 1–3 h, decreasing to 6 h
- Macrophages *anti-27E10* positive*: 2–3 h
- Neutrophils *IL-1α* positive*: evident 4 h–1 day, peak 7 h
- Neutrophils *ubiquitin* positive*: 4 h–1 day
- Neutrophils *IL-8, MCP-1* and *MIP-α* positive*: 4–12 h
- Basal layer keratinocytes with *RNA* positive for *3H-citidine**: 10–24 h
- *Non-specific esterase* (NSE)°: >1 h (enzyme-histochemistry), >5 min (microspectrophotometry)
- *ATPase*°: >1–4 h
- *Esterase*°: >2 h
- *Adenosintriphosphatase*°: >2 h
- *Acid phosphatase*°: > 2–6 h
- *Alkaline phosphatase*°: >3.5–8 h
- *Aminopeptidase*°: >4 h
- *Succinyldeydrogenase*°: >4 h
- *Lactate dehydrogenase*°: >4 h
- *6-glucosephosphatodehydrogenase*°: >4 h
- *Putrescine, cadaverine, spermidine* and *spermine:* with HPLC sudden increase at 12 h
- *C5b-9*^: >1 h
- *ICAM-1* #: evident at 1.5 h and 3.5 days. Not reliable as a single parameter
- *VCAM* #: evident >3 h, peak 4–6 h, decrease to 3.5 days

Wound age estimated in days

- *SCF-positive* mast cells*: peak 1 day
- Absence of erithrofages*: <2–3 days
- Absence of infiltration of macrophages*: <3 days
- Fibroblasts *p53* positive*: >3 days
- Fibrocytes CD45+/Col I+positive*: >4 days
- *Smooth muscle actin* positive myofibroblasts*: >5 days
- Absence of *Ki67* positive fibroblasts*: <6 days
- Macrophages *anti-RM 3/1* positive*: 6 days
- Layer of migrated keratinocytes*: >7 day
- Macrophages *anti-25F9* positive*: 11 days
- Absence of keratinocytes positive for *cytokeratin 5**: <19 days
- Mast cells *Kit receptor* positive*: peak 14 days

Table 3 (continued)

- *Collagen IV* ^: >4 days
- *Collagen III* ^: >4 days
- *Tenascin*^: if negative <5 days, affected by post-mortem phenomena
- Absence *collagen III* ^: <5 days
- Absence *collagen V* ^: <6 days
- Absence *collagen I* ^: <6–7 days
- Absence *collagen VI* ^: <6–7 days
- *Collagen III, V, VI and I* if they are present simultaneously^: lesion >7 days

Early and late markers

Considering the various and numerous studies carried out in the field of injury timing, it is worthwhile briefly reviewing the most significant results obtained to date which could be useful and of immediate consultation. Unfortunately, this does not allow reference to all pertinent manuscripts. Markers indicative both of cell and enzyme movement, as well as markers of reactions in the extracellular matrix and of endothelial adhesion molecules, are reported in Table 3 together with the information regarding time that can be considered reliable as reported in the scientific literature.

As already pointed out, the most recent studies have focused primarily on the soluble mediators of wound healing, which are expressed by all inflammatory cell types and, therefore, can provide good information concerning the grade of the wound healing stage. The value, as proof in court of the results, is, unfortunately, weaked by the approximation of data which are expressed in terms of percentage of cell type positive to one or to another mediator. Nevertheless, some information may be useful, such as, for example, the absence or insignificant presence of some markers in healthy skin, which give reliable indications, when present, regarding the vitality of the lesion, although their timing needs to be further investigated (Table 4).

Parameters always absent in injuries induced post-mortem also provide very important information in the assessment of lesion vitality, and are used in the differential diagnosis between vital and post-mortem injury (Table 5).

The unusefullness of a parameter has always to be taken into account, despite its importance as a mediator. Some markers, in fact, are considered unreliable for forensics on account of their contradictory results or the variability in their action (Table 6).

Practice advise

In the attempt to establish the chronology of an injury, the forensic pathologist has to select the appropriate markers to be identified, according to the technology available in his/her institute as well as the estimated timing of the injury inflicted (Table 3).

If the time of injury is estimated to be approximately 2–3 h, it is worthwhile attempting to identify the markers of endothelial activation (# sign in Table 3) given their reliable time of appearance and disappearance.

Wound vitality can be assessed by seeking parameters which are never or constitutionally scarsely present on healthy skin (* symbol in Table 4).

Concerning the differential diagnosis between in vivo and post-mortem injury, this can be solved by seeking those parameters which are known to always be absent in wounds inflicted post-mortem (^ symbol Table 4).

Table 4 Markers absent or less present on healthy skin (*) or always absent in post-mortem induced injuries (^)

- *IL-1β* never present on keratinocytes *. Present in the dermis and excretory ducts of sweet glands
- *TNF-α* rarely on keratinocytes *
- *IL-6* rarely on keratinocyte *
- *VCAM* rare *
- *Selectin E* never*
- *Selectin P* presence individual-dependent *
- *VAP-1* produced by damaged blood vessels. To be studied *
- *PAF* produced by damaged blood vessels. To be studied *
- *Leukotriene-β4* ^
- *Cathepsin A and D* ^
- Fibrin *D-dimer* ^
- Basal layer keratinocytes with RNA positive for *3H-cytidine* ^

Table 5 Markers considered unreliable in forensics

- *Prostaglandins*
- *Fibrin*
- *Fibronectin*
- *Cytokeratin 13*
- α1-anti-chymotrypsin and α2-macroglobulin
- *Histamine*
- *Proteases*
- *ICAM-1* if the only parameter
- *Selectin L*

Finally, the markers listed in Table 5 should not be used since they are not considered reliable.

The larger the number of markers evaluated simultaneously, the greater the possibility of defining the time of injury.

Geno-proteomics and future perspectives

Future research must focus on further evaluation of parameters scarsely present or absent in healthy skin, which represent reactions that take place only after inflammatory stimuli and thus help in establishing the timing with the least possible margin of error. Studies on artificial skin are also providing new means for collecting information as well as treatment options [88]. Furthermore, the possibility of defining to the link between the pathogenic stimulus and the first cell receptor, or between the mediator and its integrin, would notably contribute to assessing the vitality of an injury. The application of nanotechnology (proteomics, genomics and metabolomics) in the research will offer the possibility to better identify the time appearance of parameters of possible future interest (see Table 6 for some items).

Another solution could be in the development of the so-called analytical morphology. This could pave the way for a meeting point in which different skills come together each of which contributing to better timing.

IHC supported by ISH, in situ PCR, antigen retrieval, image analysis, confocal microscopy, etc., could all enable forensic histopathology to maintain its role as the gold standard for injury timing.

Furthermore, with the advent of nanotechnology, an exciting area of research is offered and forensic genetics could make an important contribution. This is, in part, thanks to the innovations provided by the further development of techniques of real-time PCR, in the study of mRNA that provides informations regarding the degree of cell activation after an inflammatory stimulus and by the investigation into the production of proteins that play a role in wound healing.

Studies on anaphylaxis using in situ PCR and real-time PCR, by amplification of RNA, exploit the opportunity of demonstrating molecules that are expressed at low levels (e.g. growth factors, receptors and developmental signals). This opens a wide field of possibilities to fully photograph the inflammatory grade at the moment of death which provide important proof in court. This technique has been used to study the expression of troponin I mRNA in contused skeletal muscle of rats [89].

Microarray analysis, using material obtained from tissues or cells, offers the possibility to analyse gene expression profiles and hence identify the degree of the reaction. In fact, the detection of mRNA, which may or may not be just translated into protein, reflect the "intentions" of the cell at the time of death, in other words, what protein the cell was about to synthesise and in what quantities. This maps the grade of inflammation which can be translated into time since injury, thus avoiding the problems related to the evaluation of the percentage of effective presence of the parameters mentioned above.

At the same time, these new advancements could offer the possibility of simultaneous and multi-parametric analysis of mediators present in serum and/or in tissues, paving the way for a more complete interpretation of the pathological phenomena and molecular mechanisms by providing information also on DNA, RNA, SNP, oligonucleotides, proteins, and peptides. Although there are still problems regarding data analysis, this approach will certainly be very worthwhile in the diagnosis of timing of injuries.

Combining proteomic and genomic technologies, such as matrix-assisted laser-desorption ionisation (MALDI) mass spectrometry (MS) with reverse transcription and in vitro transcription labelling of the resulting cDNA, it is possible to simultaneously learn both the protein production

Table 6 Parameters of possible future interest

- *VAP-1*: enzyme produced by the damaged vessels
- *PAF:* phospholipid produced by damaged endothelial vessels
- *Pattern recognition receptors (PRR)*: since these cause the inflammatory process, they could provide information on its intensity and quality
- Better understanding and development of the role of *cytokines*
- Degree of activation of *NFkB* in the cells of interest
- Further study into the phenomena within the *extracellular matrix*
- Role of hypoxia induced factor-1α (*HIF*-α)

already planned but not implemented, and that which is already in situ [90, 91].

Techniques certainly of great interest, at a proteomic level, are the MALDI time of flight (ToF) MS for protein identification by peptide mass fingerprinting, and the electrospray ionisation-ion traps (ESI-IT) MS and the nano-liquid chromatography (nano-LC) MS coupling for protein and peptide identification and characterisation.

The application of MALDI-ToF MS to incised wounds of rats demonstrates that, after wounding, the most prominent change is in the level of haemoglobin, which is elevated after 5 min and remains elevated for 3 h, falling to near control levels after 12 h [92].

Finally, the possibility of identifying the metabolites of inflammation mediators with metabolomic techniques such as gas chromatography/MS (GC-MS) system, gas chromatography/combustion/isotopic ratio MS (GC-C-IRMS) system, LC/triple-quadrupole MS (LC-QqQ) system, metabolic profiler (LC/RMN/ToF), LTQ Orbitrap, Fourier-transform ion cyclotron resonance (FT-ICR) MS and LC/quadrupole ToF (LC-QToF) MS system, will offer new diagnostic perspectives leading to the identification of those details that still remain unknown using traditional and modern techniques.

In conclusion, according to current knowledge, wound healing may be seen as a transcriptional genetic programme activated by signals of danger and damage to tissues. The tissue damage and the recognition of pathogens, as well as the cellular activation of IL-1 and TNF activate a cascade of kinases that are directed toward the "awakening" of NFkB triggering genes that encode molecules essential for initiating the inflammatory process. Therefore, the possibility to assess, without any reasonable doubt, the time of an injury is closely related to the translation of the dialogue that occurs between pathogenic stimulus and receptor on responsive cells, between cell receptors and integrines, and between integrines and effector mediators.

Forensic histopathologists will also have to understand how this dialogue is influenced by the multiplicity of intracellular and intratissular phenomena taking place during the transition between the life of the organism and cell death, defined as agony response or intermediate reaction, as well as in the post-mortem period, giving an assessment to what could be called the physiopathology of the cadaver.

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