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REVIEW ARTICLE

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Histological and enzyme histochemical parameters for the age estimation of human skin wounds

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Abstract Routine histological staining techniques form the basis of a forensic age estimation of human skin wounds and the determination of vitality is aided by the detection of neutrophilic granulocytes which appear earliest about 20–30 min after wounding. A clear granulocyte infiltration and a significant increase in the number of macrophages indicates a post infliction interval of at least several hours. Macrophages containing incorporated particles such as lipophages, erythrophages or siderophages appear earliest at a wound age of 2–3 days similarly to extracellular deposits of hemosiderin, whereas the rarely detectable iron-free pigment hematoidin and spot-like lymphocytic infiltrates in the granulation tissue appear approximately one week or more after wounding. A complete reepithelialization of surgically treated and primarily healing human skin lesions can be expected earliest 5 days after wound infliction and the absence of a complete new epidermal layer indicates a survival time of less than 21 days. Enzyme histochemical methods allow a wound age differentiation especially in the range of a few hours. An increase in nonspecific esterases can be observed earliest approximately 1 hour after wounding followed by other enzymes such as acid phosphatase (~ 2 h), ATPase (~ 4 h), aminopeptidase (~ 4 h) or alkaline phosphatase (~ 4 h). Positive results, however, cannot be regularly found. Therefore, the detection of reactive changes is useful for a wound age estimation whereas negative findings, which in general must be interpreted with caution, can provide information only in a limited number of histological parameters.

Key words Wound age · Histology · Enzyme histochemistry

Zusammenfassung Routinehistologische Färbungen bilden die Basis der forensischen Altersbestimmung menschlicher Hautwunden und erlauben durch den Nachweis von neutrophilen Granulozyten frühestens ab etwa 20–30 Minuten nach Wundsetzung Aussagen zur Vitalität einer unbekannten Verletzung. Eine intensive Granulozyten-Infiltration im Wundgebiet ist – wie auch eine relevante Vermehrung von Makrophagen – erst mehrere Stunden nach Verletzung zu erwarten. Makrophagen mit differenzierter Phagozytosetätigkeit wie Lipophagen, Erythrophagen und Siderophagen belegen – wie auch der extrazelluläre Nachweis von Hämosiderin – eine Überlebenszeit von mindestens 2–3 Tagen. Das sehr selten darstellbare Hämatoidin und das Vorliegen fleckförmiger Lymphozyten-Infiltrate im Granulationsgewebe lassen auf eine Überlebenszeit von mindestens 1 Woche schließen. Ist der ursprüngliche Defekt im Bereich der Epidermis chirurgisch versorgter und primär heilender Wunden wieder vollständig durch Keratinozyten gedeckt, belegt dies ein Wundalter von mindestens 5 Tagen, bei Fehlen einer vollständigen Reepithelialisation kann auf eine Überlebenszeit von weniger als etwa 21 Tagen geschlossen werden. Enzymhistochemische Untersuchungen sind vor allem zur Differenzierung von Überlebenszeiten im Bereich weniger Stunden vorteilhaft und eine Aktivitätszunahme der unspezifischen Esterase in Fibroblasten des Wundgebietes kann frühestens etwa 1 Stunde nach Wundsetzung beobachtet werden, gefolgt von entsprechenden Veränderungen der sauren Phosphatase nach ungefähr 2 Stunden sowie der ATPase, der Aminopeptidase und der alkalischen Phosphatase nach etwa 4 Stunden. Positive Befunde sind jedoch keinesfalls regelmäßig zu erheben und nur bei sicherem Nachweis einer Aktivitätserhöhung des jeweiligen Enzyms ist auf eine entsprechende Mindest-Überlebenszeit zu schließen. Die Ergebnisse unterstreichen die besondere Bedeutung positiver Befunde für die Wundaltersbestimmung. Das Fehlen reaktiver Veränderungen, welches unter forensischen Aspekten grundsätzlich mit Zurückhaltung interpretiert werden sollte, gibt hingegen nur bei einer begrenzten Zahl von Parametern Hinweise auf die Überlebenszeit.

Dedicated to Prof. Dr. W. Eisenmenger on the occasion of his 50th birthday

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Schlüsselwörter Wundalter · Routinehistologie Enzymhistochemie

Introduction

The timing of human skin wounds is one of the most important medico-legal problems and is based on the observation of a normal course of the wound healing process which was first described by Cohnheim in 1867 [15]. During the healing process several morphologically distinguishable phenomena occur and their microscopical detection determines the minimum wound age whereas the absence of these parameters provides less reliable results and often cannot be interpreted under forensic aspects. Since the precision of a wound age estimation increases with the number of evaluable parameters, a variety of methods have been examined which explains the immense amount of references dealing with the problem of the timing of skin wounds. Milestones for forensic pathologists are the publications of Walcher [84–86], Orsos [56], Raekallio [68, 71], Berg [6] and Janssen [37, 38]. However, many of the results reported have been obtained from experimental animals and cannot therefore be easily transferred to the human situation under forensic aspects. Furthermore, different definitions of "reactive changes" seem to be responsible for the considerable variations in the earliest appearance of morphological parameters during wound healing which have been described by different authors [for review see 52].

The present study was performed to investigate parameters useful for a forensic timing of human skin wounds in an extensive survey with special reference to the importance of a forensic wound age estimation and to the partially contradictory results reported in the literature.

Materials and methods

A total of 221 human skin wounds (lacerations, surgical or stab/cut wounds) from 148 male and 73 female corpses without signs of putrefaction (post mortem interval less than 4 days) were evaluated. The post infliction interval ranged from a few seconds to 7 months (sec–1 h: $n = 34$; > 1 h–1 day: $n = 38$; > 1 day–1 week: $n = 48$; > 1 week–7 months: $n = 101$). The individuals were aged between 15 and 94 years with an average age of 50 years (see Fig. 1). Patients showing severe malnutrition or diseases which could have influenced the wound healing process such as malignancies or metabolic disorders (for example severe diabetes mellitus) were excluded. Furthermore, no substances such as cytostatic agents or glucocorticoids were administered during therapy according to the clinical reports. Relevant in-patient treatment with the administration of other substances such as barbiturates were recorded and considered by evaluation as well as the presence of a considerable blood loss. The causes of death were traumatic (accidents, falls homicides due to sharp or blunt trauma) or natural events (myocardial infarction, intracerebral bleeding). In addition, 40 postmortem lesions (lacerations or subcutaneous bleedings due to postmortem puncture of the femoral vein) were induced on uninjured skin of selected patients and were evaluated in comparison to normal skin specimens (internal controls).

At least 3 specimens were obtained from each skin wound and paraffin sections (3–5 µm) were prepared. Cryostate sections from 47 skin lesions with a post infliction interval between 20 min and 5 days and from 20 postmortem wounds were prepared to investigate enzyme histochemical staining procedures. The paraffin sections were stained with HE and Prussian-Blue to detect neutrophils, lymphocytes, macrophages, erythrophages, siderophages, hemosiderin or hematoidin and to examine the degree of reepithelialization of the defect. The results obtained for the detection of lymphocytic infiltrates were confirmed in selected specimens by immunohistochemistry using the monoclonal antibody CD 3 which specifically recognizes T-cells (Fa. Dako, Hamburg, Germany). In addition, cryostate sections were stained with Sudan to identify lipophages which could also be clearly detected in HE-stained sections due to the typical morphology. A modified May-Grünwald-Giemsa staining was performed to detect eosinophilic granulocytes.

The enzyme histochemical procedures were restricted to the evidence of nonspecific esterases, acid and alkaline phosphatases, aminopeptidase and ATPase as described previously [13].

The parameters were evaluated by recording the earliest and latest point in time of the post infliction interval at which each parameter was detectable. Furthermore, it was investigated in which period of wound age the parameters were present in all lacerations evaluated to define a possible regular appearance. The presence of differences dependent on individual age, wound localization (head, trunk, extremities) or type of wounding (laceration – stab/cut wound – surgical wound) was also examined.

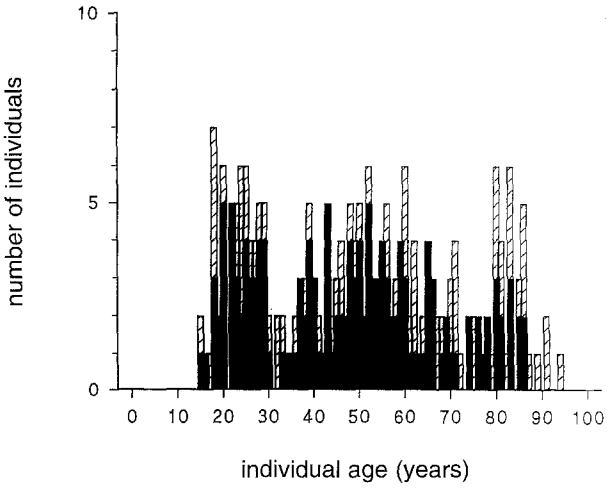

Fig. 1 Distribution of sex and individual age in 221 patients. ▨ Female ($n = 73$), ■ male ($n = 148$)

Results

I. Routine histology

Vital wounds (see Table 1 and Fig. 2)

Neutrophilic granulocytes (PMN). Positive reactions, defined as the presence of more than 10 cells outside the areas of bleeding were first detectable in skin wounds aged about 20–30 min. Reactive changes were found in all lesions with a post infliction interval of 15 h or more and in 4 out of 32 specimens (13%) with a survival time between 1 and 15 h no unambiguous infiltration was observed. The number of neutrophils decreased with increasing post infliction intervals (approximately 11 days or more) but these cells were also present (in reduced amounts) in some lesions aged more than 1 month.

Table 1 Appearance of histologically detectable parameters in human skin wounds dependent on the post infliction interval ($n = 221$)

Parameter	Earliest appearance	Regular appearance	Latest appearance
Neutrophil granulocytes	20–30 min	> 15 hrs	Months
Macrophages	3 hrs	> 3 d	Months
Macrophages > granulocytes	20 hrs	> 11 d	Months
Lipophages	3 d	(> 5 d)	Months
Erythrophages	3 d	–	Months
Siderophages/hemosiderin	3 d	(> 7 d)	Months
Hematoidin	(8 d)	–	Months
Lymphocytes	(8 d)	(> 19 d)	Months
Fibroblastic cells	~ 1 d	> 5 d	Months
Migrating keratinocytes	2 d	> 6 d	–
Complete reepithelialization (surgical wounds)	5 d	> 20 d	–

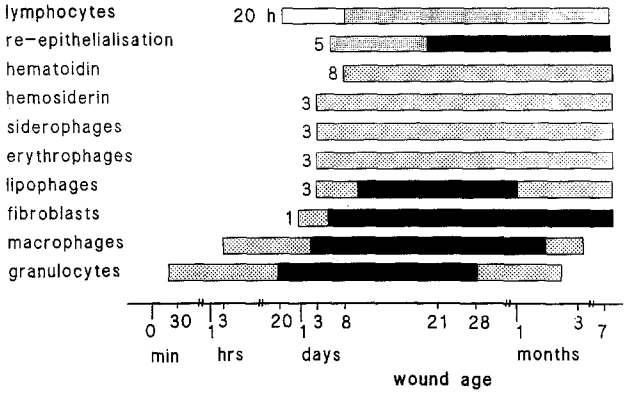

Fig. 2 Age-dependent appearance of histological parameters. ☐ Questionable, ⬚ not regular, ■ regular

Eosinophilic granulocytes. In vital skin wounds only very few of these cells were found in the area of bleeding and a questionable increase in number occurred first after a post infliction interval of 2 days. A few eosinophilic granulocytes localized outside the area of bleeding were detected earliest after a survival time of 11 days, but positive results were obtained only in a very few specimens (5 out of 39, i.e. 13%) aged up to 3 months.

Macrophages. An infiltration of macrophages was first observed in a lesion aged 3 h and was a regular finding after post infliction intervals of 15 h or more. Only one wound aged 3 days showed no clear increase in the number of cells in the wound area. Positive results were also obtained in lesions with advanced survival times up to 2.5 months and in 6 out of 10 specimens (60%) aged between 1.5 and 7 months.

Ratio of granulocytes/macrophages. In all skin wounds aged less than 15 h and showing a reactive leukocytic infiltration, the number of PMN exceeded that of macrophages but mononuclear phagocytes predominated first in a laceration with a post infliction interval of 20 h. Such findings were regularly observed after survival times of 12 days or more. The period between 2 and 11 days was characterized by a considerable variability in the ratio and the numbers of granulocytes also exceeded macrophages in a specimen with a wound age of 11 days.

Lipophages. Lipid-phagocytosing macrophages with a typical "swollen" and "clear, granular" cytoplasm were detectable earliest after a post infliction interval of 3 days and occurred in all wounds aged between 12 and 31 days. Lipophages were identified in 29 out of 35 cases (83%) with a wound age between 6 and 12 days and polynuclear lipophages were found in lesions with survival times of 5 days or more. Polynuclear as well as mononuclear lipophages were also detectable in the "oldest" wound investigated (post infliction interval 7 months).

Erythrophages. An unambiguous incorporation of red blood cells by macrophages could be observed earliest 3 days after wounding and occurred up to survival times of 2.5 months. The post infliction interval between 3 days and 2.5 months was characterized by a wide variability and positive results were obtained in 76 out of 140 lesions (54%).

Siderophages and hemosiderin. Positive reactions were found in wounds aged at least 3 days and were still observed after a post infliction interval of 7 months. Extracellular deposits of hemosiderin were found in 71 out of 76 specimens (93%) with a survival time between 3 days and approximately 2 months while siderophages were detectable in 64 out of 76 cases (84%) during this period.

Hematoidinphages and hematoidin. Iron-free degradation products of hemoglobin were found earliest after a wound age of 8 days and occurred up to survival times of 1.5 months, but positive results were found in only 5 out of 59 cases (8%).

Lymphocytes. An arbitrarily estimated but questionable increase of lymphocytes in areas of bleeding was first observed 20 h after wounding. Spot-like infiltrations occurred first in a wound aged 8 days and were commonly found in lesions with advanced post infliction intervals between 20 days and 7 months. In our series, however, there was a distinct individual variability and an unambiguous increase of lymphocytes was found in only 83 out of 154 cases

(54%) aged between 1 day and 7 months whereas 41 out of 58 wounds (71%) with a post infliction interval between 20 days and 7 months gave positive results.

Fibroblastic cells. An increased number in typical spindle-shaped cells of the infiltrate was detectable earliest in a wound with a survival time of 25 h and was observed regularly in the granulation tissue of lesions aged 6 days or more. At this point in time the regular presence of newly formed capillaries or small vessels was also recorded while the earliest appearance of typical granulation tissue was found in a wound aged 3 days. Increased numbers of fibroblasts were detected in 25 out of 46 cases (54%) with a post infliction interval between 1 and 5 days.

Epithelial cells. Large keratinocytes with a "clear" cytoplasm migrating into central parts of the epidermal defect were found first in a lesion aged 2 days. A complete reepithelialization of the former defect occurred earliest after a post infliction interval of 5 days and was found in all wounds at least 21 days old. The period between 2 and 21 days was characterized by a considerable variability and 48 out of 63 cases (76%) with a wound age between 5 and 21 days showed incomplete reepithelialization, but migrating keratinocytes were clearly identified in all specimens aged 7 days or more.

Postmortem wounds. Macrophages with incorporated particles (lipophages, erythrophages, siderophages), hemosiderin, hematoidin, migrating keratinocytes or increased numbers of fibroblastic cells were exclusively detectable in vital skin wounds with advanced post infliction intervals, but not in postmortem wounds. However, cells which usually occur in blood (e.g. neutrophilic and eosinophilic granulocytes, macrophages without phagocytosed material or lymphocytes) were sometimes found in considerable amounts in the areas of bleeding of postmortem wounds. In some of these cases, an unambiguous differentiation between postmortem artefacts (for example an accidental attachment of erythrocytes to the surface of macrophages) and initial stages of reactive cell infiltration was impossible.

II. Enzyme histochemistry

Normal skin

Nonspecific esterases. A band-shaped but weak positive staining was found between the stratum granulosum and stratum corneum. Skin appendages such as the sheath of hair roots, hair follicles, sweat glands as well as the musculi arrectores pilorum and dermal fibroblasts showed strong reactions.

ATPase. No staining was observed in the stratum corneum. A moderate reaction was detectable in the stratum granulosum and stratum basale whereas the stratum spinosum showed only a weak staining. Skin appendages, vessel walls, musculi arrectores pilorum and dermal fibroblasts, however, revealed clear positive reactions.

Aminopeptidase. Weak staining was observed in the stratum spinosum and stratum basale, skin appendages reacted strongly and dermal fibroblasts showed a moderate staining.

Acid phosphatase. Distinct positive reactions were found in stratum corneum, in skin appendages and in dermal fibroblasts.

Alkaline phosphatase. The epidermal layers showed no staining, but positive reactions were seen in skin appendages, vessel walls and dermal fibroblasts.

Table 2 Wound age-dependent increase in enzyme activity of dermal fibroblasts in human skin wounds ($n = 47$)

Enzyme	Earliest appearance	Regular appearance
Nonspecific esterases	~ 1 h	–
Acid phosphatase	~ 2 h	–
ATPase	~ 4 h	–
Aminopeptidase	~ 4 h	–
Alkaline phosphatase	~ 4 h	–

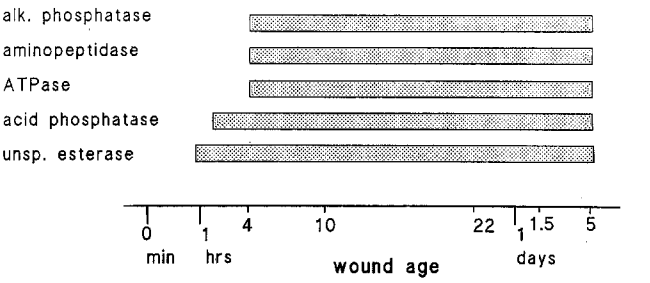

Fig. 3 Age-dependent increase in the activity of fibroblastic enzymes. ⊡ Not regular

Vital skin wounds (see Table 2 and Fig. 3)

Nonspecific esterases. An clear increase in the activity of dermal fibroblasts was first detectable in a skin wound aged 1 h and positive results could be obtained in 14 out of 35 cases (40%) with a post infliction interval between 1 h and 5 days.

ATPase. Increased activities occurred earliest approximately 4 h after wounding and only 5 out of 24 lesions (21%) with a post infliction interval between 4 h and 5 days showed positive results.

Aminopeptidase. Enhanced fibroblastic reactions were first detectable in a wound aged 4 h and were obtained in only 18% (6 out of 33 cases) with a survival time between 4 h and 5 days.

Acid phosphatase. Increased numbers of strongly reacting fibroblasts occurred first 2 h after wound infliction and positive results were found in only 6 out of 30 lesions (20%) aged between 2 h and 5 days.

Alkaline phosphatase. The earliest appearance of increased activities was recorded about 4 h after wounding and positive findings could be observed in 29% (7 out of 24) of the cases with a survival time between 4 h and 5 days.

Postmortem wounds

In postmortem injuries no increased enyzme activities were found which could be confused with positive reactions observed in vital wounds.

III. Influence of individual age, wound localization, type of wounding, blood loss and patient treatment

In our series, a clear tendency to a faster wound healing process was observed in younger individuals and in general lesions localized on the head showed an earlier appearance of reactive changes when compared to those localized on the trunk or extremities. On the other hand, there was a considerable interindividual variability and in a few skin wounds of patients with advanced individual age a very early appearance of reactive changes was found whereas in injuries localized on the head of younger patients (without intensive care, relevant blood loss or the application of pharmacological substances) the appearance of positive results was comparatively delayed. In addition, no relevant differences between lacerations, stab/cut or surgical wounds could be established. Due to these considerable variations, no grouping was possible which would have reduced the variability in the wound age-dependent appearance of positive results.

Discussion

Information on the post infliction interval of skin wounds can be obtained especially by microscopical detection of cellular reactions. Since the question of the vitality of a wound is of particular relevance in forensic medicine, the earliest appearance of parameters which occur exclusively in vital lesions is of great importance. The criteria of the regular or latest detection of parameters, which are useful in particular for the examination of injuries with long-term post infliction times, only provide information on the wound age in cases in which negative results are obtained, indicating post infliction intervals of less (or more) than the respective time intervals. On the other hand, it must be emphasized that negative findings are less reliable since the lack of microscopical evidence of reactive changes in specimens of a wound can be explained by methodological reasons or interindividual variations [7, 72] supporting the special importance of positive results for a forensically applicable wound age estimation.

With regard to these practical aspects, the microscopical timing of wounds should be based exclusively on an unambiguous detection of reactive changes in a sufficient number of specimens from each skin lesion and the examination of parameters which can occur as artefacts in postmortem injuries should be avoided. Therefore, the release of mast cell proteins as a morphological vital sign [7] seems problematic due to the difficulty of unambiguous detection. Furthermore, it is of importance that cells which are usually present in blood can drift passively into an (postmortem) area of bleeding and possibly simulate vital changes. Such a mechanism must be taken into consideration for granulocytes, macrophages (without phagocytosed particles) and lymphocytes leading to the suggestion that only the appearance of a relevant number of these cells outside the area of bleeding can be regarded as a vital sign. The question arises whether a granulocytic infiltration can be assumed if at least 3–4 of these cells are localized outside the "direct" bleeding zone [37] or the presence of a single extravascular granulocyte is sufficient to indicate an initial reaction [52]. In our opinion, only the clear evidence of neutrophils outside the area of bleeding can be regarded as the earliest reactive change which is detectable by routine histology. In our series, polymorphonuclear granulocytes (PMN) were observed first in skin wounds aged approximately 20–30 min. Our results are similar to those of Walcher [84, 86], Leder and Crespin [41], Ojala et al. [54], Prokop and Göhler [60] and Cottier [16] whereas considerably longer intervals have been reported by other authors [1, 5, 42, 43, 68, 75, 80, 87]. These differences can probably be explained by different definitions of reactive changes or by methodological reasons. Since a regular appearance of neutrophils, i.e. positive results in all wounds investigated, was observed in lesions aged 15 hrs or more, the lack of these cells in a sufficient number of specimens indicates a post infliction interval of less than 15 hrs, but cannot prove it with certainty. On the other hand, PMN were – as well as other histological criteria – present in the granulation/scar tissue of wounds with advanced post infliction intervals up to a few months in reduced amounts. These parameters, therefore allow no further differentiation of advanced wound ages especially with regard to the relationship between the extent of the wound area and the time-interval necessary for a complete restitution.

Although the detection of eosinophilic granulocytes has been suggested to be potentially useful for the timing of skin wounds [2], our investigations did not result in reliable information for a forensic wound age estimation due to the difficulty of unambiguous detection or the very irregular appearance of this parameter. Similarly, considerable differences in the initial detection of macrophages in human skin wounds have been published ranging from 2 to 24 h [7, 41, 46, 47, 50, 54]. In our series, macrophages were found outside the area of bleeding earliest 3–4 h after wound infliction, but negative results occurred up to a wound age of 3 days. A clear infiltration of macrophages generally indicates a wound age of at least 1–2 days and negative results suggest a wound age of less than 3 days.

With advanced post infliction intervals the number of granulocytes decreases while an increase in the number of macrophages take place [22, 46, 47]. The ratio between these cell types in the wound area can also be used for a wound age determination and according to Berg [5, 6] macrophages predominate after a survival time of 12–24 h or more. We observed such findings earliest in a wound aged 20 h and in all lesions with a post infliction interval of 12 days or more a preponderance of mononuclear phagocytes was seen. Since the period between 20 h and 11 days was characterized by a wide variability, the data reported by Berg [5, 6] apparently do not mean that such results can be obtained in the majority of wounds with a post infliction interval of at least 12–24 h. The relationship between the number of granulocytes and macrophages can, however, enable the differentiation of wounds a few hours or some days old. But the detection of infiltrating macrophages is not the only criteria for an indication of wound age, the presence of macrophages with different incorporated particles can also contribute to the timing of lesions. As previously described [8], lipid-phagocytosing macrophages appear at the earliest 3 days after wound infliction and can be observed almost regularly in lacerations with a post infliction interval of 5 days or more. These results support the findings of Beneke [3] who found lipophages in traumatized adipose tissue 5 days after wounding. Negative results, however, especially in skin lesions without a relevant destruction of adipocytes, could be explained by the absence of mechanically released lipids which must be phagocytosed.

Besides the incorporation of lipids, erythrophagocytosis is one of the major phagocytic functions of macrophages during wound healing. The incorporation of red blood cells is initiated by a contact between erythrocytes and the macrophage-surface and the detection of more than 3 erythrocytes attached to the macrophage is termed "rosette-formation". According to Oehmichen [52] "rosettes" occur earliest 9–11 h after extravasation and should be distinguishable from an accidental attachment of red blood cells in paraffin sections with a thickness of less than 5 µm. In our opinion, however, the formation of "rosettes" cannot be used for a forensically applicable wound age estimation due to the possible arteficial formation in postmortem wounds or lesions aged less than 9 h. Since no objective criterium exists to distinguish these accidental formations from vital ones, only the unambiguous detection of incorporated erythrocytes seems to provide reliable results. Such findings were observed earliest in our series 3 days after wounding and therefore considerably later than the interval of 15–17 h reported by Oehmichen [52]. This difference can perhaps be explained by a different definition of "erythrophages" and in order to avoid a misinterpretation of artefacts we considered only those cells to be positive which clearly showed incorporated erythrocytes localized in the same niveau as the nucleus of the macrophage. Erythrophages were detectable in many, but not all, wounds aged between 6 days and a few months indicating that only positive results prove a minimum wound age of approximately 2–3 days.

The appearance of hemosiderin and siderophages was, as expected, closely correlated to that of erythrophages since hemoglobin of extravasated erythrocytes is to a great extent degraded intracellularly by the microsomal hemoxygenase of macrophages [39, 58, 82]. Iron deposits occurred in our series at the earliest 3 days after wounding and these results support the findings of Berg [5, 6], Berg and Elbel [7], Mueller [48], Hueck [36] and Lalonde et al. [40] while other authors report a considerably delayed appearance [24, 49]. Wille and co-workers [88, 89], however, described an earliest appearance of hemosiderin 30 min after infliction of surgical wounds following staining with TP-Phloxin-B and even a postmortem formation up to an interval of 4 h after death. But these data are not easily conceivable with regard to the time necessary for the incorporation of erythrocytes and the intracellular degradation of hemoglobin to hemosiderin. Furthermore, the TP-Phloxin-B-method, which enables a differentiation between hemoglobin and hemosiderin due to different colours, does not seem to be superior to the Prussian-Blue reaction for the specific detection of hemosiderin. Additionally, an assumed postmortem formation of hemosiderin, which could not be observed in our material nor by other authors, would exclude the use of this parameter for a forensic wound age estimation.

In addition to the formation of hemosiderin, the iron-free pigment hematoidin occurs following hemoglobin degradation which can be distinguished after staining with Prussian Blue from the blue hemosiderin-deposits due to its yellow colour. The data reported for the earliest appearance of hematoidin in human skin wounds show considerable variations and range from 3 up to 43 days [7, 28, 31, 49, 84, 86]. In our series, hematoidin was first detectable 8 days after wounding and the "oldest" lesion showing positive results was aged 1.5 months. The appearance of this pigment, however, was a very rare finding, as described by other authors, and therefore only positive results provide information on wound age indicating a post infliction interval of at least approximately one week.

Lymphocytes are mainly involved in chronic inflammatory processes and their potential use as a parameter for wound age estimation has previously been discussed. Raekallio [68] and Helpap [33] found only a few lymphocytes in the wound area whereas Sieracki and Rebuck [78] described a preponderance of these cells in the exsudate 12 h after trauma. With regard to the finding of other authors [33, 68] and to our results, only the appearance of typical spot-like lymphocytic infiltrates outside the area of bleeding which indicates a wound age of at least approximately 8 days, can be used as a reliable parameter since a relevant number of lymphocytes could also be found in areas of bleeding in postmortem injuries or wounds with short post-infliction times. Such infiltrates, however, occur rather irregularly, but can be present up to survival times of a few months.

Besides the above mentioned parameters, an increase in the number of dermal fibroblasts in the wound area is useful for the timing of skin lesions. Hirvonen [34] described immigrating fibroblasts 1–2 h after wound inflic-

tion in experimental animals whereas Ross and Benditt [75], De Vito [20] and Cottier et al. [17] found an increase in number earliest about 24 h after trauma. Under forensic aspects, however, it seems difficult to unambiguously decide whether a slight increase in the number of these cells has taken place. More reliable results can only be obtained using immunohistochemical methods identifying various subtypes of fibroblastic cells which express different markers not present in normal skin [9, 10, 19, 26, 27, 79]. Following HE-staining, however, the development of a granulation tissue containing numerous typcial spindle-shaped fibroblastic and migrating endothelial cells can unambiguously be detected. In our series, such findings occurred earliest 3–5 days after wound infliction indicating a survival time of at least a few days.

In addition to the formation of a granulation tissue which fills the former defect of the dermal connective tissue, the degree of epidermal regeneration provides further information on wound age. In this context, 2 parameters are of interest, firstly the point of time at which keratinocyte migration starts and secondly the interval necessary for a complete reepithelialization. Up to now, the detection of the initial stages of keratinocyte migration or proliferation was well to the fore but parameters useful for a forensic wound age estimation could not be obtained despite using radiographic or immunohistochemical techniques [11, 12, 20, 30, 32, 45, 51–53]. In routine histology, the examination of keratinocyte proliferation does not seem possible but the beginning of epidermal migration can be detected by evidence of large flat and "clear" epidermal cells at the wound edge. In our series, such cells could be identified earliest about 2 days after injury and our results are similar to the time-interval of 1–3 days reported by Gillman et al. [30], Marks [45] and Robertson and Hodge [74]. On the other hand, the completion of reepithelialization seems to be a more reliable parameter for wound timing due to the simplicity of detection. In this context, however, the observation that the time interval necessary for a complete reepithelialization depends on the size of the defect is of particular importance. Therefore, only "standardized" epidermal defects, i.e. under practical aspects especially surgically treated and primarily healing wounds, can be compared whereas abrasions of different size cannot be examined using this parameter. Ordman and Gillman [55] investigated surgical wounds in pigs and found that the wound surface was covered by keratinocytes in 24 h while the scab was present up to 5 days. These experimental results, however, cannot be transferred to the human situation due to methodological reasons and the studies of other authors concerning the reepithelialization of (smaller-sized) injection marks in human support this view since considerably longer periods of more than 2 days have been reported [14, 25, 76]. According to our results, a complete reepithelialization occurs in surgically treated and primarily healing human skin wounds earliest 5 days after injury and is to be regularly expected in lesions aged 21 days or more. The interval between 5 and 21 days was characterized by a wide variability, but migrating keratinocytes were clearly detectable in every wound with a post infliction interval of 7 days or more. Positive or negative results, therefore, indicate a corresponding minimum or maximum survival time, respectively.

In addition, other histological parameters are discussed to be useful for the age estimation of wounds especially with advanced post infliction intervals. But the appearance of reticular fibers in lesions aged at least approximately 4 days, of van Gieson-positive fibers in wounds with a survival time of 8–10 days or more and the evidence of anisotropic fibers 56 days or more after wound infliction [24, 50] can only be regarded as indications since immunohistochemical methods allow a specific detection of the different components and therefore provide much more reliable results.

Besides routine histological techniques, enzyme histochemistry makes a substantial contribution to a morphological wound age estimation. The studies of Raekallio in particular [61–70] and Raekallio and Mäkinen [73l should be considered to elucidate the significance of these methods for the forensic timing of skin wounds. Raekallio investigated wound age-dependent changes in the activity of unspecific esterases, acid and alkaline phosphatase, aminopeptidase and ATPase in skin wounds of guinea pigs and emphasized the use of enzyme histochemistry for the evaluation of putrefied skin. Other authors studied additional enzymes such as β-glucuronidase, monoaminoxidase, cytochromoxidase, phosphorylases and succinate dehydrogenase [5, 6] and introduced these parameters into a forensic wound age determination. According to Raekallio an increase in the activity of unspecific esterases and ATPase occurs 30–60 min after injury whereas the activity of aminopeptidase increases after 2 h or more. Corresponding changes in the enzyme activity of acid and alkaline phosphatase follow 4 or 8 h after wounding, respectively, while postmortem injuries show no reactive changes [70]. These experimental results were examined in skin wounds of 43 humans and Raekallio described a "general transferability" but observed 5 exceptions with considerably delayed reactions and explained the differences in one of these 5 cases by an advanced individual age [66, 70]. On the other hand, in experimental animals Raekallio and Mäkinen found no differences in the first signs of enzyme activity changes relative to individual age [73].

In our series, the activity of the nonspecific esterases increased approximately 1 h after injury, followed by corresponding changes of acid phosphatase (~ 2 h), aminopeptidase (~ 4 h), ATPase (~ 4 h) and alkaline phosphatase (~ 4 h) whereas postmortem induced injuries showed no increased activities. Other authors also reported differences to the intervals found by Raekallio and even a postmortem increase in the activity of unspecific esterases was described [18, 59]. An assumed increase of activity in postmortem lesions, however, would limit the use of this parameter under forensic aspects even though vital and postmortem changes should be distinguishable. Regardless of a possible postmortem increase in the activity of unspecific esterases [18, 59] or differences in the earliest appearance of positive results, the opinion of Raekallio, that negative findings indicate a postmortem infliction or a

wound age less than the time interval reported for the earliest appearance, must be contradicted. Since increased enzyme activities were found only in 18–40% of our cases the critical views of other authors must be supported concerning the reliability of enzyme histochemical techniques in forensic wound age estimation [21, 35, 83] even though other studies confirmed in general the data for the earliest appearance of reactive changes reported by Raekallio [4–6, 18, 23, 29, 44, 57, 59, 77, 81]. In particular Dotzauer and Tamaska [21] emphasized that results obtained in experimental animals cannot easily be transferred to the human situation and, in addition, Berg [4] reported that the regularity of results as described by Raekallio occurred only in experimental animals but not in human skin wounds.

Therefore, it must be concluded that exclusively positive results indicate a minimum post infliction interval of a few hours. Negative results, however, are of no practical meaning due to their rather frequent appearance.

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