Int J Leg Med (1995) 107:197–200

ORIGINAL ARTICLE

P. Betz · J. Tübel · W. Eisenmenger

Immunohistochemical analysis of markers for different macrophage phenotypes and their use for a forensic wound age estimation

Received: 30 August 1994 / Received in revised form: 23 November 1994

Abstract A total of 117 vital skin wounds (post infliction intervals between a few seconds and 7 months), 20 postmortem wounds and skin specimens with beginning or advanced signs of putrefaction were investigated. Different markers for macrophage maturation (27 E 10, RM 3/1, 25 F 9, G 16/1) were analyzed by immunohistochemistry. The early stage inflammation marker 27 E 10 stained macrophages, but also monocytes and neutrophilic granulocytes localized in blood vessels or bleeding induced postmortem and therefore provided no further information for a forensic wound age estimation in comparison to the routine histological detection of macrophages. The antigens recognized by the RM 3/1– (intermediate stage inflammation marker) and 25 F 9-antibodies (late stage inflammation marker) were expressed exclusively by histiocytes and inflammatory cells that had migrated from the blood vessels as part of the acute inflammatory response associated with an intravital reaction. The morphometrical analysis revealed positive results (defined as at least a two-fold increase in number in 2 or more microscope fields when compared to the maximum value of histiocytes found in uninjured skin) for the RM 3/1– or 25 F 9-antibody earliest in wounds aged 7 or 11 days, respectively. Similarly to the 25 F 9-antibody, the chronic stage inflammation marker (G 16/1) reacted with a macrophage subpopulation first detectable 12 days after wounding but showed positive results in a comparably reduced percentage of cases. On the other hand, this marker did not stain a relevant number of resident macrophages thus facilitating the evaluation of the specimens. The markers 27 E 10, RM 3/1 and 25 F 9 are also useful for the evaluation of slightly – even though the staining intensity was considerably reduced – but not advanced putrefied skin. Therefore, the immunohistochemical analysis of the corresponding antigens can possibly contribute to an age estimation of wounds with advanced post infliction intervals obtained from corpses with longer – but limited – postmortem intervals.

Key words Macrophages · Wound age · Immunohistochemistry

Zusammenfassung Insgesamt wurden 117 vitale Hautwunden (Überlebenszeit wenige Sekunden bis 7 Monate), 20 postmortal gesetzte Verletzungen sowie Haut mit leichten bzw. fortgeschrittenen Fäulnisveränderungen untersucht und verschiedene Marker der Makrophagen-Differenzierung (27 E 10, RM 3/1, 25 F 9 und G 16/1) analysiert. Der „early stage inflammation marker" 27 E 10 färbte neben Makrophagen auch Monozyten und neutrophile Granulozyten, die innerhalb von Blutgefäßen bzw. in postmortal gesetzten Blutungen lokalisiert waren und liefert somit keine Informationen zum Wundalter, die über die Möglichkeiten des Routine-histologischen Nachweises von Makrophagen hinausgingen. Die von den Antikörpern RM 3/1 (intermediate stage inflammation marker) und 25 F 9 (late stage inflammation marker) erkannten Antigene wurden ausschließlich von Histiozyten und reaktiv eingewanderten Makrophagen exprimiert. Die morphometrische Analyse ergab positive Ergebnisse (definiert als ein mindestens zweifacher Anstieg der Zellzahl in zwei oder mehr Gesichtsfeldern verglichen mit der maximal feststellbaren Zahl an Histiozyten in unverletzter Haut) bei Verwendung der Antikörper RM 3/1 bzw. 25 F 9 frühestens 7 bzw. 11 Tage nach Wundsetzung. Ab 12 Tagen Wundalter reagierte der „chronic stage inflammation marker" G 16/1 erstmals positiv. Das Antigen ließ sich insgesamt allerdings in einem geringeren Prozentsatz der untersuchten Wunden darstellen. Vorteilhaft ist jedoch das Fehlen einer relevanten Expression durch Histiozyten, wodurch die Auswertung der Präparate erleichtert wird. Die entsprechenden Antigene lassen sich zudem in leicht – wenn auch in einer deutlich geringeren Färbeintensität –, aber nicht forgeschritten fäulnisveränderter Haut nachweisen, so daß deren immunhistochemische Darstellung gegebensfalls auch zur Beurteilung von länger überlebten Verletzungen an Leichen mit etwas fortgeschrittener Liegezeit herangezogen werden kann.

P. Betz (✉) · J. Tübel · W. Eisenmenger
Department of Legal Medicine, University of Munich, Frauenlobstrasse 7a, D-80337 Munich, Germany

Schlüsselwörter Markophagen · Wundalter · Immunhistochemie

Introduction

Forensic wound age estimation focusses in particular on the determination of wound vitality or on the examination of rather short post infliction intervals. On the other hand, a differentiation between intervals of a few days and more than one week – for example to verify repeated trauma in cases of physical child abuse – can be of great importance. The parameters suitable for the determination of post infliction intervals exceeding one week include, in particular, the presence of hematoidin or spot-like infiltrations of lymphocytes in the granulation tissue [for review see 1, 10] whereas the analysis of the epidermal reparation including the immunohistochemical detection of different basement membrane components or cytokeratins can contribute to the determination of advanced post infliction intervals in surgically treated wounds [2, 3, 7].

Recently, different markers for macrophage-phenotypes have been described [4, 11, 12] which can be analyzed by immunohistochemistry. The early stage inflammation marker 27 E 10 is a heterocomplex formation of MRP 8 and MRP 14 and contains 2 calcium-binding proteins of the S 100 protein family whereas the late stage inflammation marker 25 F 9 represents a 86 kD protein, probably a glycoprotein on the cell surface and within the cytoplasma of mature macrophages. Further information on the surface antigens 27 E 10 and 25 F 9 or on other macrophage markers such as G 16/1 or RM 3/1 is not yet available. Since it was shown that these macrophage markers are expressed during different stages of pathological processes, for example inflammation or tumor progression [5, 6, 8, 12], immunohistochemical analysis can possibly provide information useful for a forensic wound age estimation.

Material and methods

A total of 117 primarily healing surgical wounds, lacerations and stab wounds with post infliction intervals between a few seconds and 7 months were evaluated. The specimens were obtained at autopsy from individuals aged between 16 and 83 years (average 52 years) and the postmortem interval did not exceed 3 days. No therapy with possible influence on wound healing, such as the application of glucocorticoids or cytostatic agents had been performed according to anamnestical data. In addition, 20 postmortem hematoma and 20 specimens from skin with beginning (greenish discoloration but rather well-preserved microscopic structure) or advanced (loss of the epidermal layers) signs of putrefaction were investigated.

After fixation of at least 2 specimens from each wound in 4% PBS-formaldehyde solution, paraffin sections were made and stained with H & E. The immunohistochemical staining procedures were performed after enzyme pretreatment (0.1% pronase or 0.1% trypsin) using the monoclonal "early stage inflammation marker" 27 E 10 (n = 57), the "intermediate stage inflammation marker" RM 3/1 (n = 68), the "late stage inflammation marker" 25 F 9 (n = 117) and the "chronic stage inflammation marker" G 16/1 (n = 68) (Fa. Dianova, Hamburg, Germany) according to the ABC-method described by Hsu et al. [9]. The specimens stained with the RM 3/1- or the 25 F 9-antibody were evaluated by morphometry since positively reacting resident macrophages were also present in uninjured skin. The numbers of positively stained histiocytes were counted in 20 randomly selected areas of 2.5×10^{-5} cm^2 (original size of the 100-point grid: 1 cm^2, magnification: 200 ×) and compared with those found in the granulation/scar tissue of the skin wounds. Positive results were arbitrarily defined as having at least a two-fold increase in number in 2 or more microscopical fields of the lesions when compared to the maximum value of positively reacting histiocytes determined in undamaged skin. Areas of the dermis rich in skin appendages were excluded due to the frequent presence of histiocytes.

Furthermore, differences in the earliest appearance of positive results dependent on localization (head, trunk, extremities) or type (surgical wound, laceration, stab wound) of injury or on individual age were recorded.

Results

Early stage inflammation marker 27 E 10. In normal skin, neutrophilic granulocytes and monocytes localized in blood vessels or in areas of bleeding of postmortem wounds reacted positively, additionally histiocytes were specifically stained. In skin injuries, macrophages expressing the antigen recognized by the 27 E 10-antibody were found outside the area of bleeding earliest after post infliction intervals of 2–3 hrs and were detectable in our series up to wound ages of 3 months. Since leukocytes present in blood vessels or hematomas induced postmortem were also stained, the use of this marker for a forensic wound age estimation seems to be limited and therefore no further morphometrical analysis was performed.

Intermediate stage inflammation marker RM 3/1. In postmortem wounds or undamaged skin, only resident macrophages showed specific staining whereas granulocytes or other leukocytes did not react. The morphometrical analysis revealed an average number of positively stained histiocytes of 0.63 ± 0.52 in 2.5×10^{-5} cm^2 with a maximum value of 5 positive cells per defined microscopic area. A relevant increase in the number of migrated macrophages was earliest detectable in a wound with a post infliction interval of 7 days. Positively reacting macrophages were also present in the scar tissue of the oldest wound investigated (wound age 7 months) and positive results were observed in 72% (26 out of 36) of the cases with post infliction intervals between 7 days and 7 months. The average number of specifically stained cells in the "positive" specimens was about 8.34 ± 6.81 per 2.5×10^{-5} cm^2 with a maximum value of 21 cells.

Late stage inflammation marker 25 F 9. Similar to the results obtained for the RM 3/1-antibody, only migrated macrophages and histiocytes reacted positively and the average number of resident macrophages showing a specific staining was about 0.72 ± 0.61 per 2.5×10^{-5} cm^2 in normal skin with a maximum value of 7 cells. Positive results could be obtained in skin wounds earliest after post infliction intervals of 11 days and were also detectable in injuries with advanced duration (in our series up to 3 months). Of the wounds aged between 11 days and 7

Table 1 Earliest, regular (in parentheses: percentage of positive results in the interval between earliest and latest appearance) and latest appearance of macrophages positively staining for different markers in human skin wounds

Antibody	Earliest appearance	Regular appearance	Latest appearance
27 E 10	(~ 2–3 hrs)	–	(3 months)
RM 3/1	~ 7 days	– (72%)	(7 months)
25 F 9	~ 11 days	– (69%)	(3 months)
G 16/1	~ 12 days	– (49%)	(7 months)

months, 69% (38 out of 55) showed an unambiguous increase in the number of specifically stained macrophages. The mean value in the "positive" specimens was about 9.74 ± 6.20 cells per defined area and a maximum number of 28 cells in 2.5×10^{-5} cm^2 was observed.

Chronic stage inflammation marker G 16/1. In the dermal layers of normal skin, only very few histiocytes reacted specifically but no relevant numbers of positively stained resident macrophages occurred which have to be taken into consideration for evaluation.

Considerable amounts of reacting cells were detectable earliest in a wound aged 12 days and 49% (17 out of 35) of the specimens with post infliction intervals between 12 days and 7 months gave positive results. The antigen was also present in the "oldest" lesion investigated (wound age 7 months).

Putrefied skin. In skin specimens with slight signs of putrefaction and a rather well-preserved microscopic structure, histiocytes showed positive reactions for the antibodies 27 E 10, RM 3/1 and 25 F 9 even though a considerably reduced staining intensity could be observed. In specimens with advanced putrefaction (loss of the epidermis, almost total lack of nuclear staining), however, no

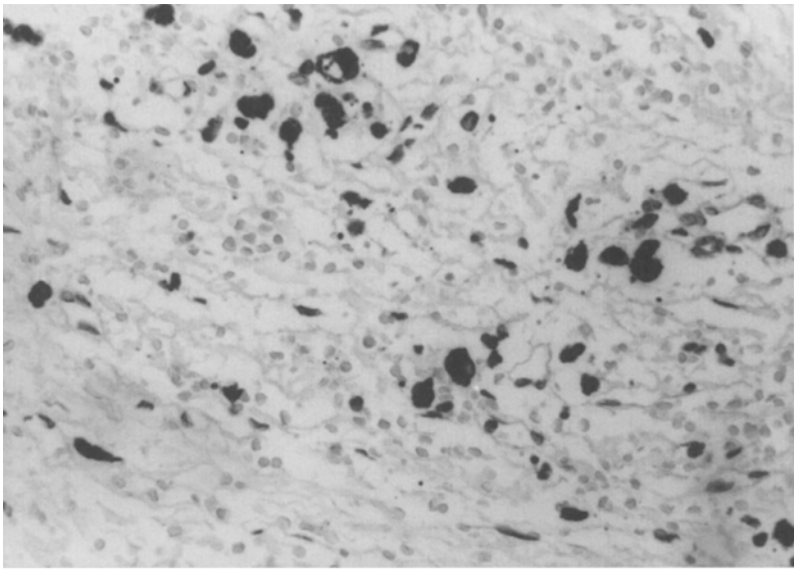

Fig. 1 Human skin wound (post infliction interval: 14 days): Numerous macrophages in the granulation tissue positively staining for the 25 F 9-antibody (paraffin, ABC, 190 ×)

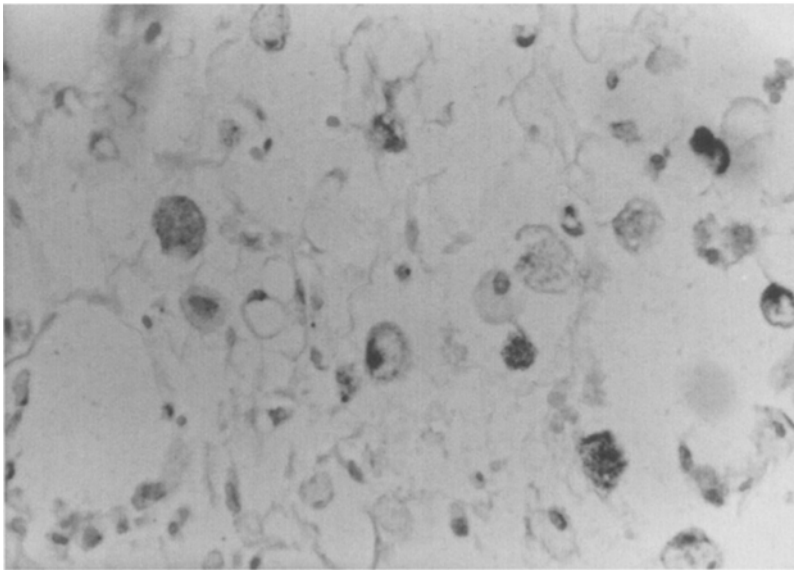

Fig. 2 Human skin wound (post infliction interval: 2.5 months): Lipid-phagocytosing macrophages positively reacting with the chronic stage inflammation marker G 16/1 (paraffin, ABC, 300 ×)

identification of histiocytes by immunohistochemistry was possible.

Relevant differences in the earliest appearance of positive results dependent on individual age, localization or type of the wounds could not be observed due to a wide interindividual variability.

Discussion

The early stage inflammation marker 27 E 10 can also be detected in monocytes and neutrophilic granulocytes limiting the use for an identification of actively migrated macrophages. Since the antigen is expressed in non-activated leukocytes localized inside blood vessels or in postmortem hematomas, cells could have passively reached into the area of bleeding and must not be interpreted as a vital sign. Therefore, the immunohistochemical analysis of this macrophage surface antigen provides no further information on wound age when compared to the routine histological detection of macrophages.

The antigens recognized by the antibodies RM 3/1, 25 F 9 or G 16/1, however, are not present on the surface of leukocytes usually occurring in blood, such as monocytes or neutrophilic granulocytes, and therefore the immunohistochemical analysis of these markers is of considerable importance. On the other hand, the antigens identified by the antibodies RM 3/1 and 25 F 9 are also expressed by histiocytes and to avoid any misinterpretation a comparison between the numbers of "physiologically" detectable histiocytes in undamaged skin and a reactive increase in skin injuries seems necessary. The morphometrical analysis showed that a relevant increase in number of positive macrophages was observed earliest in wounds aged 7 (RM 3/1) or 11 days (25 F 9), proving a corresponding minimum wound age. The accumulation of positively stained macrophages seemed to be so extensive in the granulation/scar tissue of most wounds when compared to the presence of widely dispersed single histiocytes, that positive results could also be obtained without morphometrical analysis. Such an analysis will hardly be necessary for the immunohistochemical detection of G 16/1-positive macrophages since no relevant numbers of resident macrophages expressing the recognized antigen occur. Positive results seem to be sufficient to indicate a minimum wound age of approximately 12 days but unfortunately, this marker can be observed in a rather small percentage of cases (49%) in comparison to the 25 F 9-antibody (69%). Reviewing other histological indicators for advanced postinfliction intervals, the immunohistochemical analysis of macrophage markers provides some advantages. The detection of RM 3/1-, 25 F 9- or G 16/1-expressing macrophages seems to be superior to the routine histological identification of hematoidin [10] which indicates a wound age of approximately one week or more due to the very rare appearance of this pigment. Even though spot-like infiltrates of lymphocytes in the granulation tissue are observed in a similar number of cases with postinfliction intervals of 8 days or more when compared with different macrophage markers, it can be difficult to differentiate postmortem or vital lymphocyte accumulation due to a possible passive extravasation from blood vessels. A further advantage could be that in contrast to the immunohistochemical analysis of a complete epidermal restitution, which exclusively provides information on advanced wound duration in surgically treated skin lesions [1, 2, 7], the evaluation of macrophage markers is not limited to wounds with "standardized" size. With respect to these practical aspects, the analysis of macrophage surface antigens can be regarded as a considerable complement to the limited number of parameters which can be used for the determination of advanced wound duration.

Acknowledgement This study was supported by grants from the "Deutsche Forschungsgemeinschaft" (Grant no. Be 1555/1-1 and 1-2)

References

1. Betz P (1994) Histological and enzyme histochemical parameters for the age estimation of human skin wounds. Int J Leg Med 107:60–68
2. Betz P, Nerlich A, Wilske J, Tübel J, Wiest I, Penning P, Eisenmenger W (1992) The time-dependent rearrangement of the epithelial basement membrane in human skin wounds – immunohistochemical localization of collagens IV an VII. Int J Leg Med 105:93–97
3. Betz P, Nerlich A, Wilske J, Tübel J, Penning R, Eisenmenger W (1993) The time-dependent reepithelialization of human skin wounds – immunohistochemical localization of keratins 5 and 13. Int J Leg Med 105:223–227
4. Bhardwaj RS, Zotz C, Zwadlo G, Roth J, Goebeler M, Mahnke K, Falk M, Meinhardus-Hager G, Sorg C (1992) The calcium-binding proteins MRP 8 and MRP 14 form a membrane associated heterodimer in a subset of monocytes/macrophages present in acute but absent in chronic inflammatory lesions. Eur J Immunol 22:1891–1897
5. Broecker EB, Zwadlo G, Suter C, Brune M, Sorg C (1987) Infiltration of primary and metastatic melanomas with macrophages of the 25 F 9-positive phenotype. Cancer Immunol Immunother 25:81–86
6. Broecker EB, Zwadlo G, Holzmann B, Macher E, Sorg C (1988) Inflammatory cell infiltrates in human melanoma at different stages of tumor progression. Int J Cancer 41:562–567
7. Eisenmenger W, Nerlich A, Glück G (1988) Die Bedeutung des Kollagens bei der Wundaltersbestimmung. Z Rechtsmed 100:79–100
8. Goldstein J, Braverman M, Salafia C, Buckley P (1988) The phenotype of human placental macrophages and its variation with gestational age. Am J Pathol 133:648–659
9. Hsu SM, Raine C, Fanger H (1981) A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin-complex method for studying polypeptide hormones with radio immunoassay antibodies. Am J Clin Pathol 75:734–739
10. Oehmichen M (1990) Die Wundheilung. Springer, Berlin Heidelberg New York
11. Zwadlo G, Broecker EB, Bassewitz DB, Feige U, Sorg C (1985) A monoclonal antibody to a differentiation antigen present on human macrophages and absent from monocytes. J Immunol 134:1487–1492
12. Zwadlo G, Voegeli R, Schulze Osthoff K, Sorg C (1987) A monoclonal antibody to a novel differentiation antigen on human macrophages associated with the down-regulatory phase of the inflammatory process. Exp Cell Biol 55:295–304